The results indicate that $NaBH_4$ can be used to quantitatively reduce disulfides in several proteins in aqueous solutions under relatively mild conditions. Generally, slightly milder conditions could be used for the reduction in the presence of SDS. The only difficulty with apparent reoxidation in the presence of the detergent was in the case of bovine plasma albumin where quantitative results could not be repeatedly obtained with systems containing SDS. However in this case complete reduction was obtained by treatment with $NaBH_4$ alone.

The use of $NaBH_4$ appears to have distinct advantages over other reagents commonly used for disulfide reduction in proteins: thioglycolic acid, mercaptoethanol and sulfite. Thioglycolic acid and mercaptoethanol, as any other thiols, have the disadvantage of making difficult the determination of the extent of the reduction. Additionally it has been reported recently by WHITE[6] that thiolation by polythioglycolides is a major side reaction during thioglycolate reduction of disulfide bonds in ribonuclease. The reaction of sulfite gives one sulfhydryl plus one S-sulfonate per disulfide, whereas $NaBH_4$ treatment yields two sulfhydryls. The extent of reduction by sulfite can be followed titrimetrically, but it is difficult to determine when there has been complete conversion of each half-cystine to S-sulfonate. Furthermore the reaction with sulfite also must be conducted in 8 *M* urea solutions to achieve completion[7,8].

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[1] R. CECIL AND J. R. MCPHEE, *Advances in Protein Chem.*, 14 (1959) 271.
[2] S. MOORE, R. D. COLE, H. G. GUNDLACH AND W. H. STEIN, *Intern. Congr. Biochem. 4th Meeting, Symposium No. 8, Vienna, 1958*, Pergamon Press, New York.
[3] A. M. DOLLAR, W. D. BROWN AND H. S. OLCOTT, *Biochem. Biophys. Research Communs.*, 1 (1959) 276.
[4] R. E. BENESCH, H. A. LARDY AND R. BENESCH, *J. Biol. Chem.*, 216 (1955) 663.
[5] H. C. BROWN, E. J. MEAD AND R. C. SUBBA RAO, *J. Am. Chem. Soc.*, 77 (1955) 6209.
[6] F. H. WHITE, JR., *J. Biol. Chem.*, 235 (1960) 383.
[7] J. L. BAILEY AND R. D. COLE, *J. Biol. Chem.*, 234 (1959) 1733.
[8] J. R. CARTER, *J. Biol. Chem.*, 234 (1959) 1705.

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Behavior of some selenium compounds in transmethylation

CANTONI AND MUDD[1] have shown that the selenium analogue of methionine is converted to "active selenomethionine" (Se-adenosylselenomethionine) at a rate which is similar to that at which "active methionine" is formed under identical conditions, and that when this Se-adenosylselenomethionine is incubated with guanidinoacetic acid and creatine methylpherase from pig liver, creatine is formed in excellent yield by transmethylation.

BREMER AND GREENBERG[2] have described a system in which choline biosynthesis is observed when [*Me*-^{14}C]S-adenosylmethionine is incubated with the microsome

fraction of rat liver. In the present study, the ability of Se-adenosylselenomethionine to serve as methyl donor in this system was tested using [^{3}H]selenomethionine.

Selenomethionine, prepared in this laboratory according to the procedure described by PLIENINGER[3], was randomly tritiated by WILZBACK method[4] (New England Nuclear Corp., Boston), giving the specific activity of 2.12 mC/mg. Determination of the portion of radioactivity, associated with the methyl group of [^{3}H]-selenomethionine, was carried out as follows: S-methyl isothiourea picrate was prepared from 300 mg of non-radioactive carrier selenomethionine added to $1.375 \cdot 10^6$ counts/min of [^{3}H]selenomethionine, according to the procedure described by LEVINE AND TARVER[5]. The picrate (m.p. 221° after recrystallization from ethanol) was dissolved in conc. NH_4OH, and the methyl mercaptan, liberated by gentle heating, was led into a trap containing 4 % aq. $Hg(CN)_2$. The precipitate, $(CH_3S)_2Hg$, was recrystallized from chloroform (m.p. 175–176°). 2.2 mg of the mercury compound was counted in liquid scintillation counter to give 4,580 counts/min. Calculation showed that 28.7 % of the total activity in randomly labeled [^{3}H]selenomethionine was associated with the methyl group. [^{3}H]selenomethionine was converted to Se-adenosylselenomethionine according to the procedure used by CANTONI AND DURELL[6].

Table I shows an experiment in which S-adenosylmethionine and Se-adenosyl-selenomethionine were compared as substrate in choline biosynthesis. The selenium analogue was found to be as efficient a methyl donor as S-adenosylmethionine in this system.

TABLE I

SE-ADENOSYLSELENOMETHIONINE AS METHYL DONOR IN CHOLINE BIOSYNTHESIS

Radioactive substrate (1.5 μmoles, $6.14 \cdot 10^5$ counts/min** in S-adenosylmethionine and $3.10 \cdot 10^4$ counts/min in the methyl group of Se-adenosylselenomethionine) was incubated with rat-liver microsomes*** (24 mg protein) in glycine–KOH buffer (200 μmoles, pH 9.5) for 1 h at 37° in a total volume of 2 ml. The reaction was stopped by addition of 0.2 ml conc. HCl followed by 1.8 ml H_2O and 2 ml *n*-butanol.

Substrate	*Radioactivity in butanol extract**		*Net radicactivity representing phospholipid bases*
	Intact microsomes	*Boiled microsomes*	
[*Me*-^{14}C]S-adenosylmethionine	19.4	3.6	15.8
[^{3}H]Se-adenosylselenomethionine	59.6	37.4	22.2

* Expressed as percentage of added radioactivity found in products.

** The radioactivity determination in this work was done by Packard Tri-Carb liquid scintillation counter.

*** Preparation of microsomes and determination of protein were performed as reported previously[7].

As the incubation of Se-adenosylselenomethionine with boiled microsomes gave a relatively high activity with the *n*-butanol-extraction procedure used by BREMER AND GREENBERG, the following experiment was carried out to verify the formation of [^{3}H]choline. 1 ml of the butanol extract from the incubation mixture containing intact microsomes, which had 3,350 counts/min as phospholipid bases in the extract, was evaporated to dryness and subsequently refluxed for 4–5 h in an oil bath with 3–4 ml 2.5 *N* HCl and 25 mg carrier choline. After removing the liberated fatty acids by ether extraction, the choline was precipitated as the Reineckate and recrystallized

three times from acetone with *n*-propanol. The choline was liberated from 10-mg samples of the Reineckate precipitate after each recrystallization by shaking the choline Reineckate with a mixture of 1 *N* HCl and methyl ethyl ketone. By this procedure the choline is extracted quantitatively into the aqueous phase while the liberated Reinecke salt is retained quantitatively in the methyl ethyl ketone phase. After evaporating the aqueous phase in the counting vials, the radioactivity of the choline residue was determined in the liquid scintillation counter. Specific activities of the Reineckate after one, two and three recrystallizations were 270, 245 and 253 counts/min/10 mg respectively. Considering that the molecular weight of choline Reineckate is 3.4 times that of choline chloride, at least 2,125 counts/min of the 3,350 counts in the original butanol extract must have been in the form of choline. This calculation does not take into account the unknown amount of choline present in the radioactive phospholipids extracted from the incubation mixture (presumably several mg) and it does not include the intermediate, mono- and di-methylaminoethanol, which are not coprecipitated with choline when the choline Reineckate is recrystallized.

In Table II is shown an experiment where S-adenosylmethionine and Se-adenosylselenomethionine were compared as methyl donors in the methylation of a sulfhydryl compound, 2-methoxyethanthiol, in a system identical to that used by BREMER AND GREENBERG[7]. Again, the selenium compound was found to be an effective methyl donor as estimated from the formation of radioactive toluene-soluble product, presumably O,S-dimethylmercaptoethanol.

TABLE II

Se-ADENOSYLSELENOMETHIONINE AS METHYL DONOR IN ENZYMIC METHYLATION OF A SULFHYDRYL GROUP

Radioactive substrate (0.75 μmole, $1.35 \cdot 10^5$ counts/min in S-adenosylmethionine and $1.46 \cdot 10^4$ counts/min in the methyl group of Se-adenosylselenomethionine) was incubated with rat-liver microsomes (10 mg protein) and 2-methoxyethanthiol (1 μl) in tris(hydroxymethyl)aminomethane buffer (50 μmoles, pH 8.0) at 37° in a total volume of 1ml. Reaction was stopped by addition of 0.1 ml conc. HCl and the incubation mixture was extracted with toluene.

Substrate	*Volatile radioactivity in toluene extract**		
	20 min	*40 min*	*90 min*
[*Me*-^{14}C]S-adenosylmethionine	27.2	44.1	67.0
[^{3}H]Se-adenosylselenomethionine	13.4	24.1	40.7

* Expressed as percentage of added radioactivity found in products.

CHALLENGER[8] and his group have observed that certain molds grown in culture containing inorganic selenate or selenite produce dimethylselenide, and S-adenosylmethionine has been inferred as principal methyl donor. In experiments *in vitro* with various animal tissues, conversion of inorganic selenate and selenide to "volatile selenium" has been observed[9]. BREMER AND GREENBERG[7] have shown that various sulfhydryl compounds can accept methyl group enzymically from [*Me*-^{14}C]S-adenosylmethionine. These findings led us to test H_2Se and methyl selenol as methyl acceptors in the microsomal system. As shown in Table III, these selenium compounds were found to be good methyl acceptors in enzymic methylation. The finding that some methylation takes place with boiled microsomes is in accordance with the observation

TABLE III

SELENIUM COMPOUND AS METHYL ACCEPTOR IN ENZYMIC METHYLATION

[*Me*-^{14}C]S-adenosylmethionine (0.7 μmole, 405,600 counts/min) was incubated with substrate (10 μmoles) and rat-liver microsomes (39 mg protein) in tris(hydroxymethyl)aminomethane buffer (100 μmoles, pH 8.0) for 2 h at 37° in a final volume of 1.9 ml. The reaction was stopped by addition of 0.2 ml conc. HCl. The methylation products were separated according to CHALLENGER[10]. 100–200 mg each of non-radioactive carrier, corresponding to the methylation products of the particular substrate, were added to the reaction mixture and the volatile gas was passed successively in a stream of air through (a) 4% aq. $Hg(CN)_2$ and (b) DIGINELLI's solution (10 g $HgCl_2$, 80 ml water, 20 ml conc. HCl) for 2 h with occasional swirling. 300–400 mg of the mercury salt of monomethyl compound was precipitated in (a) and 500–600 mg of the $HgCl_2$ salt of dimethyl compound was precipitated in (b). After recrystallization from benzene, the purity of the compounds was tested by melting-point determination***. In the experiment with H_2Se, the separation of the mono- and dimethyl compound was not attempted owing to the insolubility and heat lability of the methyl selenol mercury salt.

*Substrate**	*Methylation product*	*Radioactivity in methylation product***	
		Intact microsomes	*Boiled microsomes*
H_2S	CH_3SH	14.6	2.2
	CH_3SCH_3	1.8	0.4
H_2Se	$CH_3SeH + CH_3SeCH_3$	10.8	3.5
CH_3SeH	CH_3SeCH_3	11.7	3.0

* Aqueous solutions of Na_2S, Na_2Se and $NaSeCH_3$ were added as substrates.
** Expressed as percentage of added radioactivity found in products.
*** $(CH_3S)_2Hg$ m.p. 174–175°, $2(CH_3)_2S \cdot 3HgCl_2$ m.p. 157–158°, $(CH_3)_2Se \cdot HgCl_2$ m.p. 153–154° (decomp.). (These melting points are uncorrected.) These values correspond to those reported in ref. 10.

of STEKOL (private communication) that a nonenzymic transmethylation from S-adenosylmethionine to sulfhydryl compounds occurs.

The present work demonstrates that selenium analogues can replace some of the biologically important sulfur compounds in the enzymic methylation system found in rat liver.

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1 S. H. MUDD AND G. L. CANTONI, *Nature*, 180 (1957) 1052.
2 J. BREMER AND D. M. GREENBERG, *Biochim. Biophys. Acta*, 37 (1960) 173.
3 H. PLIENINGER, *Ber.*, 83 (1950) 265.
4 K. E. WILZBACH, *J. Am. Chem. Soc.*, 79 (1957) 1013.
5 M. LEVINE AND H. TARVER, *J. Biol. Chem.*, 192 (1951) 835.
6 G. L. CANTONI AND J. DURELL, *J. Biol. Chem.*, 225 (1957) 1033.
7 J. BREMER AND D. M. GREENBERG, *Biochim. Biophys. Acta*, 37 (1960) 173.
8 F. CHALLENGER, *Aspects of the Organic Chemistry of Sulphur*, Butterworth Scientific Publications, London, 1959, Chapter 5.
9 I. ROSENFELD AND O. A. BEACH, *J. Biol. Chem.*, 172 (1948) 333.
10 F. CHALLENGER, *Aspects of the Organic Chemistry of Sulphur*, Butterworth Scientific Publications, London, 1959, p. 18.

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