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Studies on the Metabolism of Unsaturated Fatty Acids. IV.[1] N-Ethylmaleimide Reducing Activity in *Escherichia coli* K-12

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An enzyme fraction which catalyzes the reduction of N-ethylmaleimide (NEM) to N-ethylsuccinimide in the presence of reduced nicotinamide adenine dinucleotide phosphate was partially purified from *Escherichia coli* extracts.

The apparent Michaelis-Menten constant for NEM was estimated to be 4 μM. The enzyme showed a rather broad pH optimum between 7 and 8.

The enzyme was 90% inhibited after being heated at 55° for 15 min, and was 94% inhibited by 1 mM *p*-hydroxymercuribenzoic acid.

Keywords——N-ethylmaleimide; N-ethylsuccinimide; N-ethylmaleimide reducing enzyme; *Escherichia coli*; NADPH

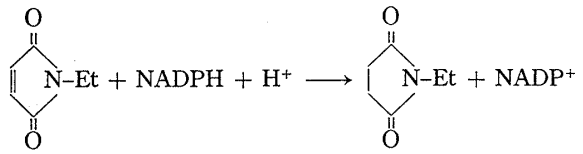
Among organic substances which bind to reactive side chains of amino acid residues in proteins, *p*-hydroxymercuribenzoic acid, iodoacetic acid, and N-ethylmaleimide (NEM) are commonly used as binding reagents for the thiol groups of cysteine residues. These compounds usually inhibit enzymatic reactions. On rare occasions, however, stimulatory effects of these compounds on certain enzymatic reactions have been reported. For example, increases of enzyme activities in the presence of NEM have been reported for enoyl-acyl-carrier-protein (enoyl-ACP) reductase[2] and for microsomal glutathione S-transferase.[3] In the case of the enoyl-ACP reductase, it was explained that NEM at a concentration of 1 mM interacted with the enzyme at a site other than the active thiol group and stimulated its activity. Microsomal glutathione S-transferase activity was stimulated strongly by treatment of the microsomes with NEM at a concentration of 1 mM, although the mechanism of its activation remains to be established.

Previously we reported the isolation of a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent *cis*-2-enoyl-coenzyme A (*cis*-2-enoyl-CoA) reductase from cell-free extracts of *Escherichia coli*[4] and of *Candida*.[5] It is assumed that the reductase participates in the β-oxidation of unsaturated fatty acids containing *cis*-double bond(s) on the odd-numbered carbon atom(s).

When the inhibitory effect of NEM on *cis*-2-enoyl-CoA reductase in cell-free extracts of *E. coli* was examined by monitoring the decrease in absorbance at 340 nm due to the consumption of NADPH, an apparent increase of the reductase activity was observed unexpectedly. Furthermore, the decrease in absorbance at 340 nm was observed even in the absence of *cis*-2-alkenoyl-CoA, if the reductase, NADPH and NEM were all present. These results suggested that NEM recieved an electron from NADPH enzymatically. The NEM reducing activity was recovered in the same fraction as that of *cis*-2-enoyl-CoA reductase on Sephacryl S-200 Superfine by gel-filtration chromatography (Fig. 1).

In order to identify the reaction product of NEM, aliquots of the incubation mixture were withdrawn at intervals, and the products were examined by gas chromatography. One peak, which was not detected at zero time, increased with increasing incubation time. It was expected to be the peak of N-ethylsuccinimide (NES) on the basis of its retention time, and its structure was determined by gas chromatography-mass spectrometry (GC–MS), as illustrated in Fig. 2. The molecular ion peaks of NEM and NES were observed at m/z 125 (Fig. 2A) and 127 (Fig. 2B), respectively.

Based on these results, the reaction catalyzed by the reducing enzyme is assumed to be as follows:



This reaction should be pH-dependent, and might be reversible at high pH values. However, all attempts to reverse the reaction either by changing the pH of the incubation medium, or by using NES and $NADP^+$ have so far been unsuccessful.

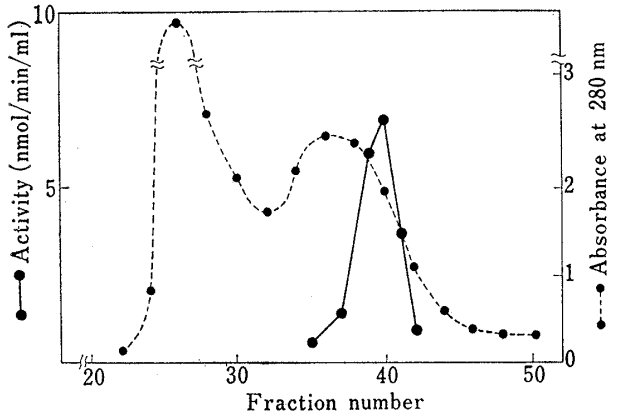


Fig. 1. Gel Filtration of Cell-Free Extracts of *E. coli* on a Sephacryl S-200 Superfine Column

Crude proteins (80 mg) were applied to the column (2.6×50 cm) and eluted with 0.02 M potassium phosphate buffer (pH 7.4). Fractions of 4 ml each were collected.

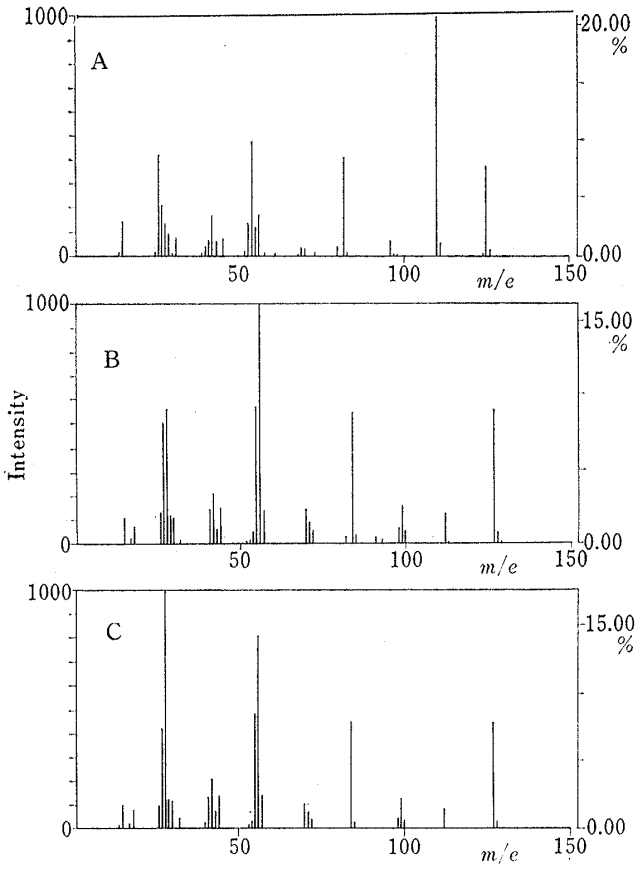


Fig. 2. GC–MS Analysis of NEM Metabolite

A: Mass spectrum of NEM (substrate); B: mass spectrum of metabolite; C: mass spectrum of an authentic sample of NES.

The enzyme from *E. coli* was partially purified by employing various chromatographic procedures, as summarized in Table I. The purity of the most active fraction after the final gel filtration was evaluated by polyacrylamide disc gel electrophoresis, which indicated the presence of at least three protein bands. Throughout the procedures employed, the NEM reducing activity was accompanied by *cis*-2-enoyl-CoA reductase activity.

From a double-reciprocal plot of the initial velocity against NEM concentration, the Michaelis-Menten constant (K_m) for NEM of the partially purified enzyme from *E. coli* was calculated to be 4 μM.

The NEM reducing enzyme showed a rather broad pH optimum between 7 and 8. The enzyme activity after treatment at 45° for 15 min was unchanged, but 90% of the activity was lost after treatment at 55° for 15 min. The NEM reducing enzyme was 94% inhibited by *p*-hydroxymercuribenzoic acid at a concentration of 1 mM, whereas 1 mM iodoacetic acid inhibited the activity about 40%. In both inhibition tests, the concentration of NEM, the substrate, was 50 μM. When NEM concentration was increased to 1 mM, a slight decrease in the reducing activity was observed, indicating inhibitory action of NEM at higher concentrations.

Table I. Purification of NEM Reducing Enzyme

Step	Specific activity (nmol/min/mg)	Purification factor (fold)
Supernatant, 105000×*g*	5.5	1
DEAE-cellulose	43.6	7.9
DEAE-Sepharose CL-6B	232	42.2
	[476]	[86.5]
Sephacryl S-200 Superfine	248	45.1
	[1353]	[246.0]

Experimental

Chemicals——NADPH, $NADP^+$ (oxidized form of NADPH), and reduced nicotinamide adenine dinucleotide (NADH) were purchased from Kyowa Hakko Co. Ltd. (Tokyo). All other chemicals were of reagent grade and were obtained from usual commercial sources.

Enzyme Preparations——*E. coli* K-12 was grown to the late exponential phase in a liquid medium containing 2% meat extract and 0.3% linoleic acid (initial pH 7.4) with vigorous shaking. The cells were collected and treated as reported previously[4b] to obtain the 105000×*g* supernatant solution. The solution (3700 mg protein) was applied to a diethylaminoethyl (DEAE)-cellulose column (3.8×29 cm) which had been equilibrated with 0.01 M potassium phosphate buffer (pH 7.4). More than 80% of the NEM reducing enzyme was eluted with 0.01 M potassium phosphate containing 0.1 M KCl. The recovered protein was applied to a DEAE Sepharose CL-6B (Pharmacia Fine Chemicals Inc.) column (2.6×29 cm) and the enzyme was eluted with a linear gradient of KCl from zero to 0.2 M in 0.01 M potassium phosphate buffer (pH 7.4). The fractions containing high enzymatic activity were combined. Approximately 70% of the activity was recovered. The concentration of potassium phosphate was adjusted to 0.1 M with the 1 M buffer and ammonium sulfate was added to 80% saturation. The precipitated protein was collected by centrifugation, redissolved in a minimum volume of 0.01 M potassium phosphate buffer (pH 7.4) and passed through a Sephacryl S-200 Superfine (Pharmacia Fine Chemicals Inc.) column (2.6×51 cm) with the same buffer as the eluting solvent.

Assay for NEM Reducing Enzyme——The enzymatic decrease in absorbance of NADPH at 340 nm was measured with a Gilford 250 recording spectrophotometer. A typical reaction mixture contained 40 nmol of NEM, 125 nmol of NADPH, 40 μmol of potassium phosphate (pH 7.4), and water to give a final volume of 0.8 ml. The reaction was initiated by adding a suitable amount of enzyme solution.

GC–MS——A JEOL JMS-01SG-2 instrument coupled with a JEOL JMA-2000 mass data analysis system was employed. The column was a 2 mm inner diameter×1 m glass coil with 5% OV-17 on Chromosorb W-HP, 80—100 mesh. The column and injection temperatures were kept at 150° and 250°, respectively. The ionizing voltage was 20 eV.

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Influence of Blood Proteins on Biomedical Analysis. II.[1] Interaction of Gliclazide with Bovine Serum Albumin

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The interactions of gliclazide, a potential hypoglycemic drug, with native and chemically modified bovine serum albumin (BSA) were studied by means of an equilibrium dialysis technique. The Scatchard plot for the interaction of gliclazide with native BSA was a hyperbola, suggesting the existence of two (or more) classes of gliclazide-binding sites on the BSA molecule ($n_1=0.5$, $K_1=160\times10^3\,\text{M}^{-1}$; $n_2=4.5$, $K_2=4.4\times10^3\,\text{M}^{-1}$). Total binding capacity ($\sum n_iK_i$) for gliclazide-BSA binding was lower ($99.8\times10^3\,\text{M}^{-1}$) than that ($190.7\times10^3\,\text{M}^{-1}$) for tolbutamide. Modification of BSA with hydrogen peroxide, iodoacetic acid, iodoacetamide or 2-hydroxy-5-nitrobenzyl bromide lowered the binding affinity in the primary binding site or destroyed the binding site, and decreased the total binding capacity of gliclazide-BSA binding. Since the binding capacities of the primary and secondary binding sites varied upon modification of cysteine, methionine, histidine and tryptophan residues of the BSA molecule, it is possible that the binding sites are both closely associated with loops 1—4 of the BSA molecule.

Keywords——hypoglycemic drug; gliclazide; bovine serum albumin (BSA); equilibrium dialysis; Scatchard plot; binding capacity for gliclazide-BSA binding; gliclazide-BSA binding; BSA binding parameter; chemically modified BSA

It has been established that the binding of sulfonylureas to circulating proteins, such as albumin or globulin, in the living body can influence their therapeutic activities.[2] In particular, the serum albumin can interact strongly with many sulfonylureas[3] and other drugs.[4] It has been confirmed to be one of the main carrier proteins for drugs.[4] It has been accepted that the pharmacological activity of sulfonylureas is potentiated by competitive displacement by other drugs, such as salicylic acid[5] or sulfonamides,[6a] from the binding sites on serum albumin. The characterization of the drug-binding sites on the serum protein, including estimation of the binding affinities, is pharmacologically important. Moreover, knowledge of the intrinsic protein-binding properties in the blood of drugs having a narrow