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Studies on Nitro-Reducing Systems of Rat Liver[1]

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Nitro-reducing systems of rat liver were studied.

1. Rat liver possessed at least two different nitro-reducing systems; they were distinguished from each other by requirement of hydrogen donor, intracellular localization and sensitivity for phenobarbital induction.

2. Microsomal reduced nicotinamide-adenine dinucleotide phosphate-linked nitro-reducing system was obtained in soluble form by the treatment of microsomes with steapsin, and was partially purified. Some properties of the solubilized system were studied.

3. Evidence was presented that microsomal NADPH-cytochrome c reductase was included in microsomal nitro-reducing system. However, the nitro-reducing system required cofactors. One of them might be flavin mononucleotide interacting weakly with protein. Effect of flavins to the nitro-reducing system was kinetically discussed.

Although only a few aromatic nitro-compounds are used as drugs, a number of them are used as materials in chemical industry and are poisonous for health of persons employed in the manufactory. It is considered that an obvious toxity of aromatic nitro-compound is partly due to the formation of methemoglobin, and its formation may be due to the reductive intermediates of nitro-group.[3]

It is well known that aromatic nitro-compound is reduced in mammalia to the corresponding amino-compound,[4] and there are several reports on enzymatic mechanisms of nitro-reducing system.[5-8] However, there are many uncertainties on the enzymatic mechanism and the exact mechanism is not known in detail.

The present paper describes studies on the enzymatic reduction of *p*-nitrobenzoic acid in rat liver. It will be shown that rat liver possesses at least two different nitro-reducing systems; they were distinguished from each other by requirement of hydrogen donor, intracellular localization and sensitivity for phenobarbital induction. Evidence will be presented that the nitro-reducing system reported by Kamm and Gillette[7] may be an unusual one. This paper also reports solubilization and partial purification of microsomal reduced nicotinamide-adenine dinucleotide phosphate (NADPH)-linked nitro-reducing system, and evidence will be presented that microsomal NADPH-cytochrome c reductase is involved in this system.

Materials and Methods

Materials——Nicotinamide-adenine dinucleotide (NAD) and nicotinamide-adenine dinucleotide phosphate (NADP) were obtained from Sigma Chemical Company. Glucose-6-phosphate (G-6-P) was obtained

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1) Parts of this work were presented at the 86th Annual Meeting of Pharmaceutical Soceity of Japan, Sendai, October 1966 and the 87th Annual Meeting of Pharmaceutical Society of Japan, Kyoto, April 1967. This work was supported in part by a Grant in Aid for Fundamental Scientific Research from the Ministry of Education.
2) Location: *Kita-12, Nishi-6, Sapporo.*
3) M. Kiese, *Pharmacol. Rev.*, **18**, 1091 (1966).
4) R.T. Williams, "Detoxication Mechanisms," Chapman and Hall Ltd., London, 1959, p. 410.
5) E. Bueding and N. Jolliffe, *J. Pharmacol.*, **88**, 300 (1946).
6) J.R. Fouts and B.B. Brodie, *J. Pharmacol. Exptl. Therap.*, **119**, 197 (1957).
7) J.J. Kamm and J.R. Gillette, *Life Sci.*, **2**, 259 (1963).
8) S. Otsuka, *J. Biochem.* (Tokyo), **50**, 85 (1963).

from Sigma Chemical Company and C.F. Boehringer and Soene GmbH. Flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) were kindly gifted by Tōa Eiyō Co., Ltd. Purified cytochrome c from yeast was kindly gifted by Sankyo Co., Ltd. *p*-Nitrobenzoic acid was obtained from Daiichi Pure Chemicals Co., Ltd. and recrystallized from benzene. Glucose-6-phosphate dehydrogenase (G-6-PDH) was purified from baker's yeast by the method of Kornberg and Horecker.[9] Alcohol dehydrogenase (ADH) was purified from baker's yeast by the method of Okunuki.[10]

Preparation of Microsomes——Rats (male Wister–King–A strain was used) were killed, liver was immediately removed, and homogenized with four parts of isotonic (0.15 M) KCl in a glass homogenizer equipped with a Teflon pestle. The homogenate was centrifuged at $10000 \times g$ for 10 minutes in a refrigerated centrifuge, and the precipitate was discarded. The supernatant was then centrifuged at $105000 \times g$ for 60 minutes in a Hitachi model 40P preparative ultracentrifuge. The resulting pellet of microsomes was resuspended in equal volume of isotonic KCl and centrifuged again as above. The washed microsomes thus obtained were finally suspended in isotonic KCl, so that 1 ml of suspension contained the microsomes from 1 g of liver (about 20 mg of protein per ml of suspension).

Solubilization and Partial Purification of Nitro-Reducing Activity——Solubilization of nitro-reducing activity from microsomes was effected by treatment of microsomes with steapsin.

Microsomes (10 to 15 mg of protein per ml) were incubated with 0.1% (final concentration) of steapsin (Sigma Chemical Company) in 0.15 M KCl–0.05 M potassium phosphate buffer (pH 8.0) at 0° for 16 hours. By this treatment, the nitro-reducing activity became unsedimentable at $105000 \times g$.

To 100 ml of the solubilized fraction, 35.1 g of solid ammonium sulfate was added (55% of saturation), and the resulting precipitate was removed by centrifugation. Further 16.5 g of ammonium sulfate was added to the supernatant (75% of saturation), and the resulting precipitate was collected by centrifugation and dissolved in minimum volume of 0.15 M KCl–0.05 M potassium phosphate buffer (pH 8.0). This fraction was used as the "crude nitro-reducing system."

For further purification, 1 ml of the "crude nitro-reducing system" was applied to a column (1.2×50 cm) of Sephadex G–200 (Pharmacia) which had previously been equilibrated with 0.15 M KCl–0.05 M potassium phosphate buffer (pH 8.0). Protein was eluted from the column with the same buffer; flow rate being 2.5 ml per hour. The fractions containing the nitro-reducing activity were combined as the "G–200 fraction."

Assay of Nitro-Reducing Activity——*p*-Nitrobenzoic acid (*p*-NBA) was used as the substrate, and nitro-reducing activity was determined by measuring the amount of *p*-aminobenzoic acid (PABA).

The reaction mixture usually contained the followings in 2.5 ml of 0.1 M potassium phosphate buffer (pH 8.0): 1.25 μmoles of *p*-NBA, a "NADPH generating system" (0.5 μmole of NADP, 5 μmoles of G-6-P and approximately 0.1 unit of G-6-PDH) and enzyme preparation. Various other substances such as inhibitors and cofactors were added as described in "Results". When NADH was used as hydrogen donor, the "NADPH generating system" was replaced by a "NADH generating system" (0.5 μmole of NAD, 200 μmoles of ethanol and approximately 10 units of ADH). Reaction was carried out in a Thunberg tube at 37° in nitrogen atmosphere. Reaction was stopped by addition of 0.5 ml of 30% trichloroacetic acid. After centrifugation, the deproteinized supernatant was subjected to determination of PABA.

Into 2 ml of the supernatant, 0.2 ml of 0.2% $NaNO_2$, 0.2 ml of 1% ammonium sulfamate, and 0.1 ml of 0.5% N-(1-naphthyl)ethylenediamine dihydrochloride were added at intervals of 10 minutes rspectively. After standing for 20 minutes at 30°, the resulting pink colored diazo dye was extracted into 5 ml of isoamyl alcohol and estimated spectrophotometrically at 540 mμ.

Assay of NADPH-Cytochrome c Reductase Activity——The activity of NADPH–cytochrome c reductase was estimated by the increase in absorbancy at 550 mμ causing reduced form of cytochrome c with a recording-spectrophotometer. The reaction mixture usually contained the followings in 3.0 ml of 0.1 M potassium phosphate buffer (pH 8.0): 0.2 μmole of ferricytochrome c, the "NADPH generating system" (the same as that used in the nitro-reducing activity), 10 μmoles of NaCN and enzyme preparation. Reaction was initiated by the addition of enzyme preparation, and followed for 30 seconds; the rate of reaction was linear with time for more than 60 seconds.

Analytical Methods——FAD and FMN contents were determined fluorometrically by the method of Bessey, *et al.*[11] Protein was determined by the method of Lowry, *et al.*[12] with bovine serum albumin as the standard.

Results

1. Preliminary Observations

In 1957, Fouts and Brodie[6] reported that nitro-reducing enzyme was present both in microsomes and in soluble fraction of liver cell, and required NADPH as hydrogen donor.

9) A. Kornberg and B.L. Horecker, "Methods in Enzymology," Vol. I, Academic Press Inc., New York 1955, p. 324.
10) K. Okunuki, "Jikken Kagaku Kōza," Vol. XXIV, Maruzen, Tokyo, 1958, p. 416.
11) O.A. Bessey, O.H. Lowry, and R.H. Love, *J. Biol. Chem.*, **180**, 755 (1949).
12) O.H. Lowry, N.J. Rosenbrough, A.L. Forr, and R.T. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

On the other hand, in 1961, Otsuka[8] reported the nitro-reducing enzymes that required NADH as the specific hydrogen donor. To determine the specificity of hydrogen donor requirement, a few experiments were performed.

When the reduction of *p*-NBA was examined with rat liver homogenate, both of NADPH and NADH were available for hydrogen donor. Fig. 1 shows the intracellular distribution of nitro-reducing activities. This result shows that rat liver possesses at least two different nitro-reducing systems, the one localizing in microsomal fraction and the other localizing in soluble fraction. The microsomal activity required NADPH as the specific hydrogen donor, and when NADH was used, little activity was observed in microsomes. This observation somewhat differs from that reported by Fouts and Brodie[6]; they had described that NADH showed the activity about one-fourth of NADPH. This discrepancy may be due to differences in the animal species and in the fractionation methods, and may not be essential. For the activity of soluble fraction, on the other hand, both NADPH and NADH were available for hydrogen donor.

Fig. 2 shows the inducible effect of phenobarbital on some enzymes of rat liver. Rats (male Wister-King-A strain, 45 to 60 days old were used) were injected *i.p.* with 80 mg of phenobarbital per kg of body weight once every 24 hours. After three injections, there was about a threefold increase over controls in the NADPH-linked nitro-reducing activity together with NADPH-cytochrome c reductase activity and with aniline hydroxylating activity, but there was not significant change in the NADH-linked nitro-reducing activity.

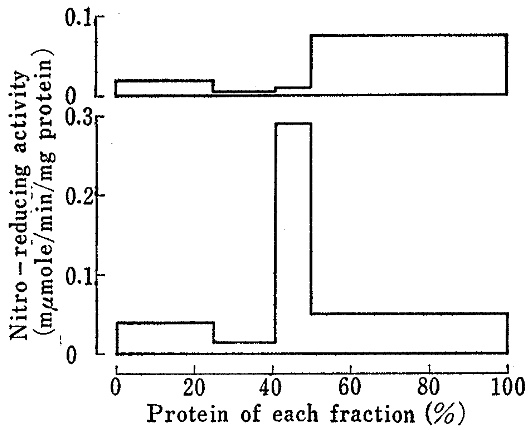

Fig. 1. Intracellular Distribution of Nitro-Reducing Activity

Liver was homogenized with 9 volumes of 0.25 M sucrose and fractionated into four subcellular fractions, *i.e.* nuclei, mitochondria, microsomes and soluble fraction, by differential centrifugation according to the method of Hogeboom and Schneider[15] with slight modification; microsomes were sedimented at 105000 × *g* for 60 minutes, and were washed with 0.15 M KCl in order to minimize adsorbedp rotein from soluble fraction.

Nitro-reducing activity of each fraction was determined as in text.

Horizontal axis shows the protein content of each fraction in percentage to the total of four fractions. The fractions are, from left to right, nuclei, mitochondria, microsomes and soluble fraction.

Vertical axis shows the specific activity of nitro-reducing activity.

upper: NADH was used as the hydrogen donor.
lower: NADPH was used as the hydrogen donor.

It is well known that phenobarbital induces microsomal drug-metabolizing activities,[13] and Hart, *et al.*[14] reported that phenobarbital has the inducible effect on microsomal NADPH-linked nitro-reducing activity of rabbit liver. The result in Fig. 2 is in agreement with that of Hart, *et al.*[14] and suggests the relationship of microsomal drug-metabolizing enzyme sytem to theNADPH-linked nitro-reducing system.

Since the nitro-reducing activity localizing in soluble fraction was lower than that localizing in microsomes, and the former was not seemed major system, this system was not further investigated.

The optimal pH of the nitro-reducing system localizing in microsomes was 7.8 to 8.2 both in Tris-HCl and potassium phosphate buffers, and the activity depended upon the ionic strength of the reaction mixture. The effects of pH and ionic strength of the mixture were similar to those of microsomal NADPH-cytochrome c reductase.[16,17]

13) A.H. Conney, *Pharmacol. Rev.*, **19**, 317 (1967).
14) L.G. Hart, R.H. Adamson, R.L. Dixson, and J.R. Fouts, *J. Pharmacol. Exptl. Therap.*, **137**, 103 (1962).
15) G.H. Hogeboom and W.C. Schneider, "The Nucleic Acids," Vol. 11, ed. by E. Chargaff and J.N. Davidson, Academic Press, New York, 1955, p. 105.
16) C.H. Williams Jr. and H. Kamin, *J. Biol. Chem.*, **237**, 587 (1962).
17) A.H. Phillips and R.H. Langdon, *J. Biol. Chem.*, **237**, 2652 (1962).

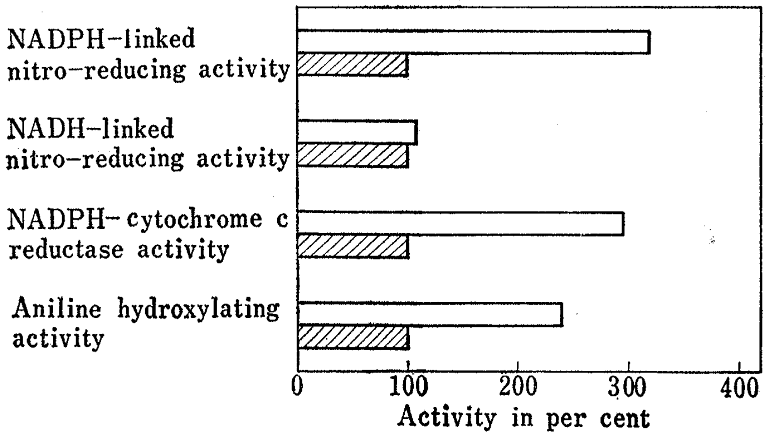

Fig. 2. Effect of Phenobarbital Injection on Some Enzymes of Rat Liver

open bar: treated rats. hatched bar: control rats
Each value is the mean of ten rats.
Treated rats were injected *i.p.* with 80 mg of phenobarbital per kg of body weight once every 24 hours for three times. Control rats were injected *i.p.* with 0.9% NaCl.
In this experiment, mitochondrial supernatant was used as the enzyme preparation.

2. Solubilization of the Nitro-Reducing Activity

Kamm and Gillette[7] reported that nitro-reducing activity in microsomes was destroyed by treatment with purified pancreatic lipase, however the solubilized fraction with pancreatic lipase treatment reduced *p*-NBA to PABA in the presence of FAD (10^{-4} M).

The authors obtained microsomal nitro-reducing activity in solubilized form without significant loss of activity by treatment with relatively high concentration of steapsin (see "Materials and Methods"). Fig. 3 shows the effect of steapsin concentration to the solubilization of the nitro-reducing activity and NADPH-cytochrome c reductase.

Microsomes were treated with various concentrations of steapsin in 0.15 M KCl–0.05 M potassium phosphate buffer (pH 8.0) at 0° for 16 hours. The suspension was then centrifuged at 105000 × *g* for 60 minutes, and the activities both in the solubilized fraction and in the residual microsomal particles were determined.

The most characteristic fact observed in Fig. 3 was that the nitro-reducing activity disappeared in parallel with NADPH-cytochrome c reductase from the microsomal particles. However, as shown in Fig. 3, the nitro-reducing activity appeared into the solubilized fraction with somewhat higher concentration of steapsin than in the case of NADPH-cytochrome c reductase, and the nitro-reducing activity seems to be partially destroyed by the treatment with 0.01% of steapsin. Similar results were obtained when trypsin was used instead of steapsin.

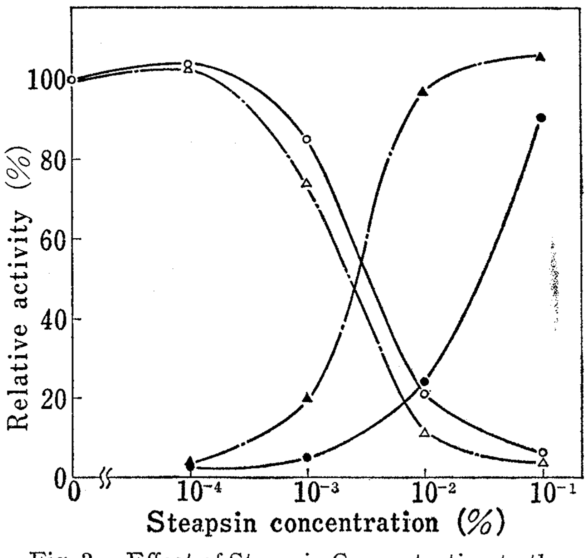

Fig. 3. Effect of Steapsin Concentration to the Solubilization of Microsomes

Microsomes were treated with various concentrations of steapsin at 0° for 16 hours. The solubilized fraction and the residual particles were separated by centrifugation at 105000 × *g* for 60 minutes. Microsomes

—○— : nitro-reducing activity in particles
—△— : NADPH-cytochrome c reductase activity in particles
—●— : nitro-reducing activity in solubilized fraction
—▲— : NADPH-cytochrome c reductase activity in solubilized fraction

3. Partial Purification of the Nitro-Reducing Activity

The specific activity of nitro-reducing system in microsomes was increased two- or three-fold by solubilization. An attempt for its further purification was made by fractionation with ammonium sulfate and by gel-filtration with Sephadex G-200.

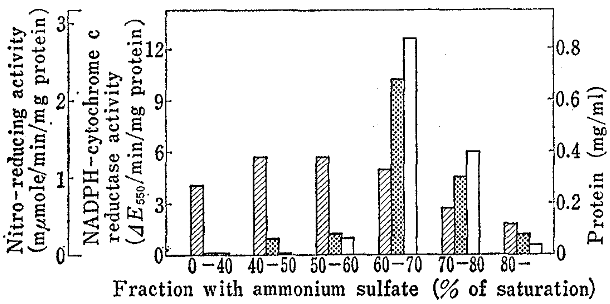

Fig. 4. Fractionation of the Nitro-Reducing Activity and NADPH-Cytochrome c Reductase Activity with Ammonium Sulfate

Hatched bar: protein content. dotted bar: nitro-reducing activity. open bar: NADPH-cytochrome c reductase activity

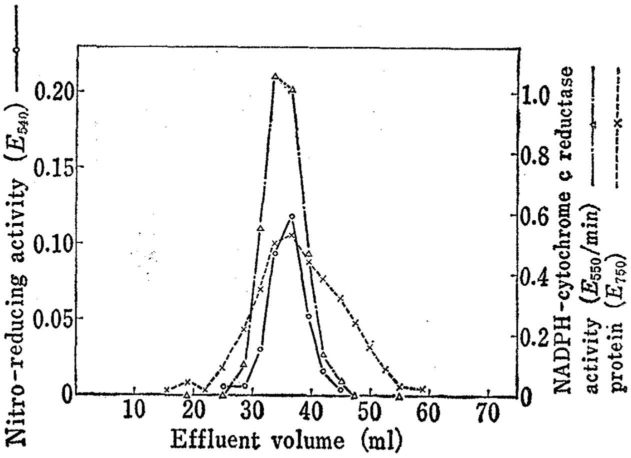

Fig. 5. Gel-Filtration of the Nitro-Reducing Activity and NADPH-Cytochrome c Reductase Activity on Sephadex G-200

Conditions are described in text.

Most of the nitro-reducing activity was sedimented at 60 to 80% of saturation of ammonium sulfate as shown in Fig. 4. The result of gel-filtration with Sephadex G-200 is shown in Fig. 5. The result of partial purification according to the procedure described in "Materials and Methods" is summarized in Table I.

TABLE I. Partial Purification of Nitro-Reducing Activity

Fraction	Protein	Nitro-Reducing Activity			NADPH-Cytochrome c Reductase Activity		
	mg	Sp. Act. mμmole/min/mg protein	Yield %	Purity	Sp. Act. ΔE_{550}/min/mg protein	Yield %	Purity
Whole microsomes	424	0.41	100	1	1.18	100	1
Steapsin sol. fraction	120	0.97	68	2.4	4.04	97	3.4
Ammonium sulfate step	12.6	3.11	22	7.7	16.8	42	14.2
G-200 step	4.7	2.12	6.0	5.3	36.1	35	30.6

As shown in Fig. 4 and 5, the nitro-reducing activity was found only in the fractions which contained NADPH-cytochrome c reductase activity. The purification of the nitro-reducing activity, however, was not in parallel with that of NADPH-cytochrome c reductase, and the nitro-reducing activity was inactivated gradually during the purification. Although a change in the ratio of two enzymatic activities during the purification usually suggests that two enzymes or enzyme systems are different, the possibility remains that NADPH-cytochrome c reductase may be involved in any step of the nitro-reduction. Since the nitro-reducing activity found only in the fractions with NADPH-cytochrome c reductase activity, the partial loss of the nitro-reducing activity during the purification might be due to loss of some cofactors, activators or additional enzymes necessary for nitro-reduction.

4. Some Properties of the "Crude Nitro-Reducing System"

Since the highest specific activity of nitro-reduction was obtained at the ammonium sulfate fractionation step and its activity was lost by further purification (see Table I), the enzyme preparation obtained at this step was used for studies of some properties of the solubilized nitro-reducing system.

Table II. Effects of Various Inhibitors

Addition	Inhibition (%)	
	Nitro-reducing Activity	NADPH-cytochrome c reductase activity
None	none	none
PCMB (10^{-6} M)	none	none
PCMB (10^{-5} M)	40	10
PCMB (10^{-4} M)	100	100
PCMB (10^{-4} M)+GSH (10^{-4} M)	100	100
PCMB (10^{-4} M)+GSH (10^{-3} M)	33	none
$CuCl_2$ (10^{-5} M)	20	none
$CuCl_2$ (10^{-4} M)	100	100
NaCN (10^{-2} M)	23	none
EDTA (10^{-3} M)	28	none
Quinine sulfate (6×10^{-5} M)	31	none
Quinine sulfate (3×10^{-4} M)	59	none

The "crude nitro-reducing system" (0.33 mg of protein for nitro-reducing activity; 0.07 mg of protein for NADPH-cytochrome c reductase activity) was used.

a) Heat Lability of the Activity: The "crude nitro-reducing system" containing 1.3 mg of protein per ml of 0.15 M KCl-0.05 M potassium phosphate buffer (pH 8.0) was incubated at 45°, sampling at various periods, and the nitro-reducing activity and NADPH-cytochrome c reductase activity were determined. The result is shown in Fig. 6.

b) Effects of Some Inhibitors: The effects of some inhibitors on the nitro-reducing activity and NADPH-cytochrome c reductase activity in the "crude nitro-reducing system" are summarized in Table II. Both of the activities were strongly inhibited by sulfhydryl reagents such as *p*-chloromercuribenzoic acid (PCMB) and $CuCl_2$, indicating the essential role of sulfhydryl group in both of the activities, and this was supported by the fact that reduced glutathione (GSH) restored the activities inhibited by PCMB. The inhibition of the nitro-reducing activity by these reagents was somewhat greater than that of NADPH-cytochrome c reductase activity.

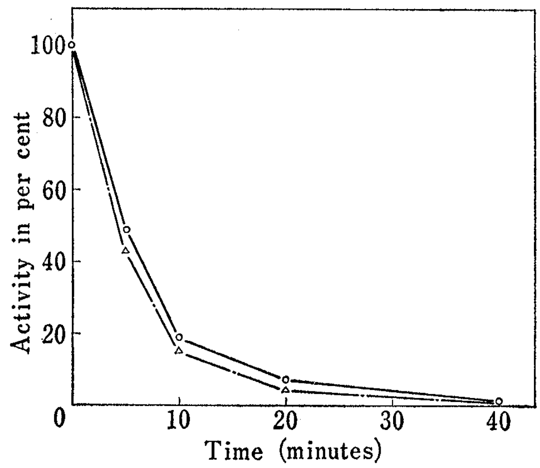

Fig. 6. Heat Lability of Nitro-Reducing Activity and NADPH-Cytochrome c Reductase Activity in the "Crude Nitro-Reducing System"

The "crude nitro-reducing system" containing 1.3 mg of protein per ml of 0.15 M KCl-0.05 M potassium phosphate buffer (pH 8.0) was incubated at 45°.
—○— : nitro-reducing activity
---△--- : NADPH-cytochrome c reductase activity

Since the nitro–reducing activity was inhibited little by metal binding reagents such as NaCN and EDTA even in high concentrations, it is supposed that the nitro–reducing system does not involve metal as essential factor.

The inhibition of the nitro–reducing activity by quinine is to be noted, because quinine is believed to compete with flavin nucleotide. Although the NADPH–cytochrome c reductase is a flavoprotein containing FAD as the prosthetic group,[18] no inhibition was observed by quinine. No inhibition of quinine to NADPH–cytochrome c reductase may be due to the strong interaction of FAD to the apoenzyme of the NADPH–cytochrome c reductase. It may be supposed that the nitro–reducing activity requires a flavin nucleotide as a activator interacting weakly with protein.

c) Effect of Dialysis and Role of FMN on the Nitro–Reducing Activity: The effect of dialysis on the nitro–reducing activity, NADPH–cytochrome c reductase activity and content of flavin nucleotide of the "crude nitro–reducing system" is summarized in Table III. Dialysis was performed by passage of the sample through a column of Sephadex G–25.

Table III. Effects of Dialysis on Flavin Content and Enzyme Activity

Preparation	Nitro–reducing activity mμmole·min⁻¹ mg protein⁻¹	NADPH–cytochrome c reductase activity ΔE_{550} min⁻¹ mg protein⁻¹	Flavin content FMN mμmole·min⁻¹ mg protein⁻¹	Flavin content FAD mμmole·min⁻¹ mg protein⁻¹
Nondialyzed	3.55	14.8	3.7	0.75
Dialyzed	2.14	20.8	2.1	0.89

The "crude nitro–reducing system"(1.98 mg of protein per ml) was passed through a column(2×25 cm) of Sephadex G–25, and subjected to dialyzed preparation (1.53 mg of protein per ml). By this treatment, NADPH–cytochrome c reductase activity and FAD content were somewhat increased in protein base. This may be due to removal of low molecular weight substances positive in protein determination.

Table IV. Effect of Flavins on Dialyzed Preparation

Preparation	Addition	Nitro–reducing activity mμmole/min/mg protein
Nondialyzed	none	2.68
Dialyzed	none	0.60
Dialyzed	FMN (10^{-7} M)	2.39
Dialyzed	FMN (10^{-6} M)	2.67
Dialyzed	FMN (10^{-5} M)	7.50
Dialyzed	FAD (10^{-7} M)	1.68
Dielyzed	FAD (10^{-6} M)	2.39
Dialyzed	FAD (10^{-5} M)	6.63

Dialyzed preparation was prepared by gel–filtration with Sephadex G–25. Flavin content of each preparation was follows: 0.65 mμmole of FAD per mg of protein and 2.78 mμmoles of FMN per mg of protein for nondialyzed preparation; 1.18 mμmoles of FAD per mg of protein and 2.10 mμmoles of FMN per mg of protein for dialyzed preparation. The reason for increase in FAD content is shown in note of Table III.
0.55 mg of protein for nondialyzed preparation and 0.20 mg of protein for dialyzed preparation were used.

The nitro–reducing activity and the content of FMN were apparently decreased by the dialysis, but NADPH–cytochrome c reductase activity and the content of FAD, which was the prosthetic group of the NADPH–cytochrome c reductase,[18] were not decreased by the same treatment.

These observations agree with the inhibitory effect of quinine, and it is supposed that the weakly interacting flavin nucleotide may be FMN. The role of FMN on the nitro–reducing

18) R. Sato, T. Omura, and H. Nishibayashi, "Oxidases and Related Redox Systems," Vol. II ed. by T.E. King, M. Morrison, and H.S. Mason, John Wiley and Sons Inc., New York, 1964, p. 861.

activity was shown by the effect of added flavin nucleotides to the dialyzed preparation (Table IV). In this case, both of flavin nucleotides were effective, and FMN was more effective than FAD, especially at lower level of concentration.

When concentrations of both of flavin nucleotides were increased, the nitro-reducing activity increased more greater. This may be due to the activating effect of flavins reported by Fouts and Brodie,[6] and Kamm and Gillette.[7]

5. Effect of Flavin Nucleotide

Fouts and Brodie[6] reported that microsomal nitro-reducing activity was significantly activated by the addition of flavins in relatively high concentrations (10^{-4} to 10^{-3} M). The mechanism of the activating effect of flavins was reported by Kamm and Gillette.[7] Using purified pancreatic lipase, they solubilized the enzyme system which reduced *p*-NBA to PABA in the presence of 10^{-4} M of FAD. They also found that both the solubilized enzyme system and untreated microsomes could reduce added FAD, and that $FADH_2$ rapidly reduced *p*-NBA nonenzymatically. From these observations, they suggested that flavins acted as the carrier between the substrate and the reductase, and latter step of the reactions, *i.e.* the reduction of the substrate by $FADH_2$, was nonenzymatic reaction.

In early parts of the studies of authors,[19] they obtained almost the same results as that of Kamm and Gillette.[7]

In Fig. 7, the kinetical properties of the nitro-reducing system are shown in the presence or absence of added FAD.

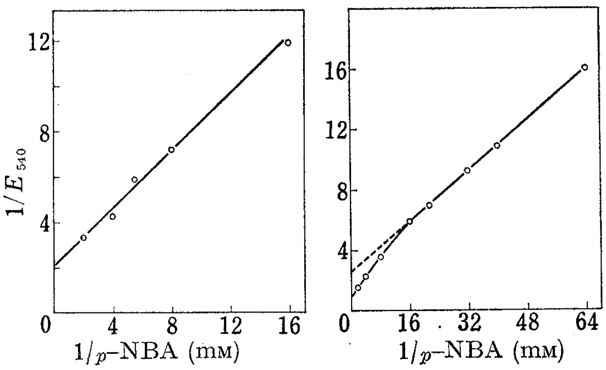

Fig. 7. Lineweaver-Burk Plot of Microsomal Nitro-Reducing Activity

A(left): without FAD B(right): with 10^{-4} M of FAD
In both experiments, microsomes (3.7 mg of protein) were used; other conditions are in text.

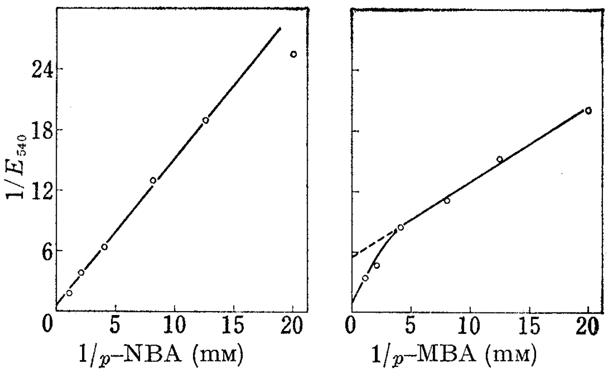

Fig. 8. Lineweaver-Burk Plot of Solubilized Nitro-Reducing Activity

A(left): without FMN. Solubilized fraction (1.32 mg of protein) was used; other conditions are in text.
B(right): With 10^{-6} M of FMN. Solubilized fraction (0.66 mg of protein) was used and incubation was carried out for 15 minutes; other conditions are in text.

The initial rates of *p*-NBA reduction were obtained, with untreated microsomes as the enzyme preparation, for various concentrations of *p*-NBA in the presence or absence of FAD (10^{-4} M). These data were plotted according to the method of Lineweaver and Burk.[20] When FAD was not added, the straight-line Lineweaver-Burk plot was obtained as shown in Fig.7A. The apparent Michaelis-Menten constant for *p*-NBA was 2.7×10^{-4} M. On the other hand, when 10^{-4} M of FAD was added, the Lineweaver-Burk plot was bent downward by the increasing concentration level of *p*-NBA as shown in Fig. 7B. In general, such kind of plot is obtained when two enzymes act on one substrate with different affinity. In this case, when the substrate concentration is sufficiently low, the straight-line Lineweaver-Burk plot is obtained, and its extention cuts the base-line at $-1/K_{m_1}$, corresponding to the Michaelis-Menten con-

19) These studies were presented at the 38th Annual Meeting of the Japanese Biochemical Society, Fukuoka, October 1965 (*Seikagaku*, **37**, 636 (1965)). In these experiments 0.03% of steapsin was used for solubilization of microsomes; under this condition, NADPH-cytochrome c reductase was completely solubilized but nitro-reducing activity was almost destroyed.

20) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

stant for one enzyme which has higher affinity for the substrate. As the substrate concentration level increases, the plot bends downward, and its imagenary extention cuts the base-line at $^{-1}/K_{m_2}$, corresponding to the Michaelis-Menten constant for another enzyme which has lower affinity for the substrate. The apparent two Michaels-Menten constants obtained by this way from Fig. 7B were 8×10^{-5} M and 2.5×10^{-4} M respectively.

This observation suggests the presence of two nitro-reducing systems in microsomes in the presence of FAD. It is assumed that one of these two systems is an alternative one introduced by the addition of FAD, and this system does not usually present in microsomes. It is also assumed that the alternative system corresponds to the system reported by Kamm and Gillette.[7] This assumption was supported by the same experiment using a solubilized fraction;[21] when 10^{-4} M FAD was added, the straight-line Lineweaver-Burk plot was obtained, and the apparent Michaelis-Menten constant for *p*-NBA was calculated to be 7×10^{-5} M. This system corresponded to the nitro-reducing system reported by Kamm and Gillette.[7] Therefore, the nitro-reducing system reported by Kamm and Gillette[7] may be an unusual one.

Fig. 8 shows the Lineweaver-Burk plot of the solubilized nitro-reducing activity. The apparent Michaelis-Menten constant for *p*-NBA obtained from Fig. 8A (1.3×10^{-3} M) was significantly greater than that obtained from Fig. 7A (2.7×10^{-4} M). This result seems to suggest that the solubilized nitro-reducing system may be modified one. While 10^{-6} M of FMN was added, the Lineweaver-Burk plot bent downward as shown in Fig. 8B. This result shows that the alternative nitro-reducing system corresponding to the system reported by Kamm and Gillette[7] is introduced even with 10^{-6} M of FMN, and that the solubilized system somewhat differs from the system reported by Kamm and Gillette.[7]

Discussion

Hepatic nitro-reducing system is able to use both NADPH and NADH as the hydrogen donor for reduction. This paper present the evidence for the existence of at least two different nitro-reducing systems in rat liver. They were distinguished from each other by intracellular localization, requirement for hydrogen donor and sensitivity for phenobarbital induction. The microsomal NADPH-linked nitro-reducing system corresponds to that reported by Fouts and Brodie,[6] and another one localizing in soluble fraction seems to correspond to the nitro-reducing enzymes isolated by Otsuka[8] from swine liver. He extracted the enzymes in soluble form with cold water from liver homogenate. Although the nitro-reducing enzymes isolated by Otsuka[8] from swine liver required NADH as the specific hydrogen donor, the nitro-reducing system localizing in soluble fraction of rat liver did NADH and NADPH as hydrogen donor. Therefore, if the contamination of microsomes to soluble fraction is negligible, it may be supposable that two nitro-reducing systems are present in soluble fraction. On this problem, however, further investigations were not performed by the authors.

The microsomal NADPH-linked nitro-reducing activity was solubilized by the treatment with relatively high concentration of steapsin, and was partially purified by the fractionation with ammonium sulfate. The fact that the nitro-reducing activity was removed from microsomal particles in parallel with NADPH-cytochrome c reductase activity by the steapsin digestion (Fig. 3) strongly suggests the essential participation of NADPH-cytochrome c reductase in the microsomal nitro-reducing system, because the solubilizing effect of steapsin is highly specific for NADPH-cytochrome c reductase, especially in low concentration.[22] The result obtained from phenobarbital induction agrees with this view. The participation of NADPH-cytochrome c reductase is also suggested by following facts: *i.e.* the nitro-reducing activity was found only in the fractions with NADPH-cytochrome c reductase activity during

21) 0.03% of steapsin was used for solubilization, and this system had little nitro-reducing activity unless FAD was added. See also the foot-note 19.
22) H. Nishibayashi, T. Omura, and R. Sato, *Biochim. Biophys. Acta*, 67, 520 (1963).

the purification (Fig. 4 and Fig. 5); heat inactivation and effects of inhibitors (except quinine) were almost same for both of the activities (Fig. 6 and Table II).

Although the participation of NADPH–cytochrome c reductase in the nitro–reducing activity is evident, this enzyme does not solely catalize the reduction of nitro–compound. Evidence for requirement of additional factors was shown in the data of partial purification (Table I). From the results of inhibitory effect of quinine and of the effect of dialysis on the "crude nitro–reducing system," it may be suggested that one of these additional factors is FMN interacting weakly with protein. Furthermore, the solubilization of the nitro–reducing activity required higher concentration of steapsin than that of NADPH–cytochrome c reductase (Fig. 3) suggests the presence of another components which participate in the nitro–reducing system.

In 1957, Fouts and Brodie[6] reported the activating effect of flavins to the microsomal nitro–reducing system, and in 1963, Kamm and Gillette[7] reported the mechanism of the action of FAD. In the latter report, they described that one of nitro–reducing activity in liver microsomes had been reconstituted with NADPH–cytochrome c reductase and relatively high concentration of exogenous FAD. The observation by the authors about the role of FMN seems to be similar to their system. It may be presumable that endogenous free FMN liberated from microsomes by the treatment with steapsin acts with the same manner as the exogenous FAD reported by Kamm and Gillette.[7]

The kinetical data suggest that the nitro–reducing system reported by Kamm and Gillette[7] is an unusual one, and this system showed higher affinity than the microsomal one for *p*-NBA. On the other hand, the affinity of solubilized nitro–reducing system for *p*-NBA was considerably lower than that of microsomal system. When 10^{-6} M of FMN was added to this system, an alternative system, which possessed higher affinity for *p*-NBA, appeared, and this system may also corresponds to that reported by Kamm and Gillette.[7] Hence, the solubilized nitro–reducing system described in this paper differs from that reported by Kamm and Gillette,[7] but the reactivating effect of FMN for the dialyzed preparation was not clearly distinguished from the additional effect of flavins reported by Fouts and Brodie,[6] and Kamm and Gillette.[7]

Since the affinity of the solubilized nitro–reducing system for *p*-NBA is considerablly different from that of microsomal system, the solubilized nitro–reducing system obtained by the authors may be modified one; this point being investigated.

It is interest that in Fig. 3, the pattern of appearance of the nitro–reducing activity into solubilized fraction from microsomes by the treatment of steapsin is nearly the same one as that of cytochrome b_5.[23] Furthermore, from the fact that the endogenous cytochrome b_5 in solubilized fraction is reduced by NADPH[24] it will be suggested that cytochrome b_5 participates in the solubilized nitro–reducing system. This point is also being investigated.

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23) Y. Yoshida, H. Kumaoka, and M. Akagi, unpublished observation.
24) J. Modirzadeh and H. Kamin, *Biochim. Biophys. Acta*, **99**, 205 (1965).