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Nicotinamide *N*-oxide formation by rat liver microsomes

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Nicotinamide *N*-oxide was isolated and identified first in urine of mice injected with nicotinamide-[7-^{14}C] by Bonavita *et al.* [1] in 1961. Its formation from nicotinamide by rat liver homogenates [2] and by liver microsomes of rats pretreated with phenobarbital [3] has also been reported.

Nicotinamide causes a typical type II spectral change as a result of interaction with cytochrome P-450 [4]. Nicotinamide has also been shown to inhibit aminopyrine *N*-demethylation and aniline hydroxylation by liver microsomes of normal rats [5] and phenobarbital-pretreated mice [6]. These results suggested the possibility that nicotinamide may be a substrate for the microsomal cytochrome P-450-containing monooxygenase system and may be oxidized to a corresponding oxygenated metabolite such as *N*-oxide.

In this communication, we describe the formation of nicotinamide *N*-oxide by rat liver microsomes and the inhibition of its formation by aniline and aminopyrine, substrates of the cytochrome P-450-dependent monooxygenase system, and by carbon monoxide and SKF-525A, known inhibitors of cytochrome P-450.

Materials and methods

Materials. Aniline and nicotinamide were purchased from Nakarai Chemicals, Ltd., and aminopyrine from the Aldrich Chemical Co. NADP$^+$ and glucose-6-phosphate were obtained from the Oriental Yeast Co., Ltd., and glucose-6-phosphate dehydrogenase from Boehringer-Mannheim. Nicotinamide *N*-oxide was prepared as described by Taylor and Crovetti [7]. SKF-525A (*β*-diethylaminoethyl-diphenyl propylacetate) was donated by Smith, Kline & French Laboratories, Tokyo, Japan.

Preparation of microsomes. Four- to 5-week-old male Wistar rats (specific pathogen free) were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Japan. Under ether anesthesia, livers were perfused with ice-cold physiological saline prior to excision. Homogenates were prepared in 4 vol. of ice-cold 0.15 M KCl and centrifuged at 10,000 *g* for 15 min. The decanted supernatant fraction was then centrifuged at 105,000 *g* for 60 min. The microsomal pellet was suspended in 0.15 M KCl containing 10 mM EDTA, and the suspension was centrifuged again at 105,000 *g* for 60 min. The washed microsomes were suspended in 0.1 M potassium phosphate buffer (pH 7.4).

Enzyme assay methods. Incubation mixtures consisted of microsomes (1.25 mg or 2.5 mg protein), NADP$^+$ (1 μmole), glucose-6-phosphate (5 μmoles), glucose-6-phosphate dehydrogenase (0.25 unit), $MgCl_2$ (6.25 μmoles), EDTA (0.175 μmole), sodium phosphate buffer (pH 7.4, 56 mM) and a substrate (aniline, 0.5 mM; aminopyrine, 10 mM; nicotinamide, 50 mM) in a final volume of 1.25 ml. The mixtures were incubated at 37° for 15 min for aniline hydroxylation, for 5 min for aminopyrine *N*-demethylation, and for 2 hr for nicotinamide *N*-oxidation. Aniline hydroxylase and aminopyrine *N*-demethylase were assayed by the method of Imai *et al.* [8] and Nash [9] respectively. Microsomal protein was determined by the method of Lowry *et al.* [10].

Identification of nicotinamide N*-oxide.* After enzyme reaction was terminated by heating in a boiling water bath for 10 min, an aliquot (100 μl) of the incubation mixture was applied on Toyo No. 51 paper which was then developed with three different solvent systems: solvent I, *n*-butanol (water saturated); solvent II, *n*-butanol (water saturated)–acetic acid (30:1); and solvent III, *n*-butanol (3% aqueous ammonia saturated). Spots were identified by ultraviolet absorption and by Holman reagent [11]. Nicotinamide *N*-oxide was also identified by retention time in high performance liquid chromatographic analysis described below.

Measurement of nicotinamide N*-oxide.* An aliquot of the reaction mixture was subjected to silica gel thin-layer chromatography (silica gel 60 F254) and developed with *n*-butanol (water saturated)–acetic acid (30:1). The fraction containing nicotinamide *N*-oxide was extracted from the TLC plate with methanol. The methanol extract was evaporated *in vacuo* and dissolved in a known volume of water. The water solution was subjected to high performance liquid chromatography. The apparatus used was a Twincle pump with a UVIDEC-100 III monitor and a VL-611 injector (Japan Spectroscopic Co., Ltd.). A Finepak SIL c18-5 column (4.6 × 250 mm), supplied by the Japan

Table 1. Identification of a reaction product by paper chromatography and high performance liquid chromatography

	Paper chromatography (R_f value)			High performance liquid chromatography (Retention time in min)
	Solvent I*	Solvent II*	Solvent III*	
Authentic				
Nicotinamide	0.64	0.65	0.57	24
Nicotinamide *N*-oxide	0.23	0.30	0.20	8
Reaction mixture				
0-hr Incubation	0.62	0.63	0.56	†
2-hr Incubation	0.63 0.24	0.63 0.28	0.56 0.19	8†

* Solvent I, *n*-butanol (water saturated); solvent II, *n*-butanol (water saturated)–acetic acid (30:1); and solvent III, *n*-butanol (3% aqueous ammonia saturated).

† High performance liquid chromatography was performed as described in Materials and methods.

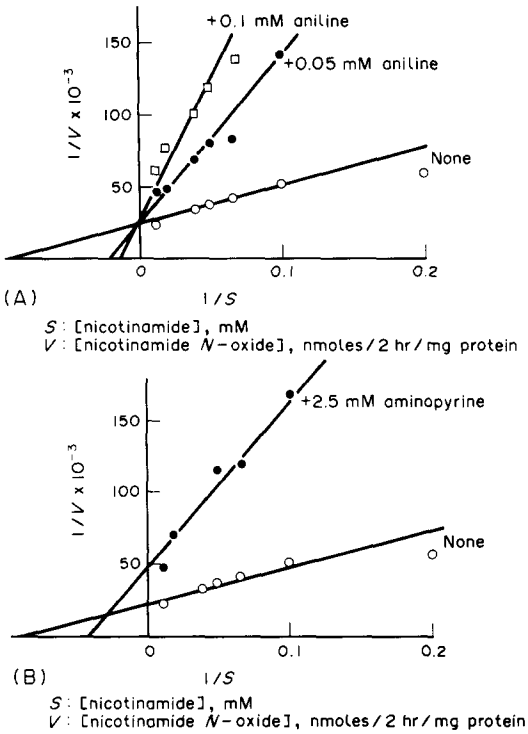

Fig. 1. Lineweaver–Burk plots showing the effects of (A) aniline and (B) aminopyrine on nicotinamide *N*-oxidation by rat liver microsomes.

Spectroscopic Co., Ltd., was used. The eluting solvent was a mixture of 0.1 M sodium phosphate buffer (pH 6.0)–methanol (95:5). Absorbance was monitored at 260 nm.

Results and discussion

When nicotinamide (50 mM) was incubated in the reaction mixture described under Materials and methods, formation of nicotinamide *N*-oxide was demonstrated by paper chromatography, using three different solvent systems, and by high performance liquid chromatography (Table 1). *N*-Oxide formation could not be detected in the absence of NADP(H). The rate of *N*-oxide formation was dependent on the concentration of microsomal protein and the length of incubation. After a 2-hr incubation with 2 mg of microsomal protein in the standard assay mixture, a total of about 80 nmoles of nicotinamide *N*-oxide was formed. The apparent K_m for nicotinamide was 11 mM.

As can be seen in Fig. 1, aniline and aminopyrine inhibited *N*-oxidation of nicotinamide by rat liver microsomes. Inhibition by aniline was competitive, while that by aminopyrine was of mixed type. Schenkman *et al.* [5] and Sasame and Gillette [6] reported that nicotinamide inhibited aminopyrine *N*-demethylation and aniline hydroxylation. In our present study using microsomes from untreated rat liver, nicotinamide inhibited aniline hydroxylation competitively and aminopyrine *N*-demethylation non-competitively or in mixed-type fashion depending on the amount of nicotinamide added to the reaction mixture.

Introduction of 30% carbon monoxide into the atmosphere of the reaction inhibited the *N*-oxidation of nicotinamide by 52%. The same concentration of carbon monoxide reduced the activity of aniline hydroxylation and that of aminopyrine *N*-demethylation by 41 and 56% respectively (Table 2). With a 25 μM concentration of SKF-525A, the activity of nicotinamide *N*-oxidation was diminished by 65%. Both aniline hydroxylation and aminopyrine *N*-demethylation were inhibited by the same percentage as nicotinamide *N*-oxidation was by SKF-525A (Table 2).

Blaauboer and Paine [3] reported that *N*-oxide formation from nicotinamide could not be detected with microsomes isolated from control rat liver. If the microsomal fraction is where *N*-oxidation occurs, it is to be expected that microsomes from control rat liver would be able to oxidize nicotinamide, for nicotinamide *N*-oxide is a normal urinary metabolite of nicotinamide in rats. In the present study, the authors have confirmed the formation of nicotinamide *N*-oxide, which was inhibited by the presence of aniline or aminopyrine, both of which are substrates for cytochrome P-450s. Carbon monoxide and SKF-525A inhibited *N*-oxide formation to the same extent as aniline hydroxylation and aminopyrine *N*-demethylation. These results strongly suggest that nicotinamide is oxidized to *N*-oxide by cytochrome P-450(s).

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Table 2. Inhibition of drug metabolism and nicotinamide *N*-oxidation in rat liver microsomes by CO and SKF-525A*

	Inhibition (%)		
Inhibitor	Aniline hydroxylation	Aminopyrine *N*-demethylation	Nicotinamide *N*-oxidation
CO	41	56	52
SKF-525A	66	61	65

* Aniline (0.5 mM), aminopyrine (10 mM), or nicotinamide (50 mM) was added as a substrate. SKF-525A was added in a 25 μM concentration.

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Anti-inflammatory factors in human synovial fluid

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Many inflammatory exudates show an anti-inflammatory activity when tested against animal models [1]. In this communication we have examined human rheumatoid synovial fluid since this is an exudate produced by a human inflammatory disease rather than by an irritant in an animal model.

Method. Human rheumatoid synovial fluid (sera-positive R.A.) was aspirated from patients at the Royal National Hospital for Rheumatic Diseases (Bath, U.K.). After aspiration the samples were centrifuged to remove cells (8000 *g* for 25 min at 4°) and stored at −30° until used. Carrageenan-induced oedema in the rat paw [2] was used to assess anti-inflammatory activity. The rats were injected i.p. with 1 ml of pooled synovial fluid 1 hr before the injection of carrageenan (0.05 ml) (2% w/v in 0.9% saline) into the hind foot. Controls were treated with saline. The oedema in the injected foot was measured 6 hr after the injection of the carrageenan by a plethysomographic method [3]. The irritancy [4] of solutions and fluids tested for anti-inflammatory activity was determined by injecting

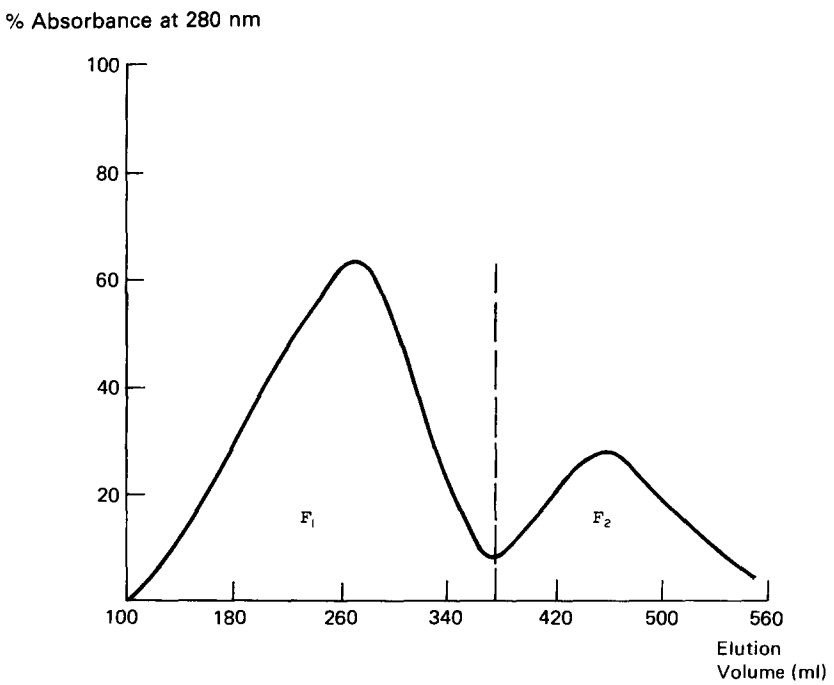

Fig. 1. The protein elution pattern of human rheumatic synovial fluid through a Sephadex G-150 column in 0.1 M phosphate buffer (pH 7.4). The absorbance at 280 nm of the eluted proteins was measured. Fractions pooled are indicated by F_1 and F_2.