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MUTAGENICITY STUDIES OF NITRAZEPAM AND ITS METABOLITE IN SALMONELLA/ MICROSOME TEST

R. L. GUPTA, GIRDHARI LAL, T. R. JUNEJA, M. S. S. MURTHY*, K. B. ANJARIA* AND N. SHANKARANARAYANAN*
Department of Pharmaceutical Sciences, Punjab University, Chandigarh 160 014, India.
*Division of Radiological Protection, Bhabha Atomic Research Centre, Trombay, Bombay 400 085, India.

NITRAZEPAM and its metabolite 2-amino-5-nitrobenzophenone were tested for their possible mutagenic activity in Salmonella/microsome test developed recently[1] to detect the environmental mutagens *in vitro*. The test employs *Salmonella typhimurium* strains as the sensitive indicators for DNA damage and mammalian liver microsomal and cytosolic fractions (S-9 fraction) for metabolic conversion of the carcinogen to their mutagenic action. Many of the nitro derivatives have been found to be mutagenic[2] in this short-term technique. The demonstration of the carcinogenicity of nitrofuran[3,4] (furylfuramide, a food preservative), nitroimidazole[5,6] (niridazole, an anti-schistosomal drug and metronidazole, a trichomonacidal drug) led us to investigate nitrazepam, a frequently used anti-convulsant drug, and its metabolite for its mutagenic activity.

For the biological activity of nitroaromatic or heteroaromatic compounds reduction of nitro group by hepatic nitro reductases present in microsomes and cytosols[7,8] appears to be essential. The reduction pathway of nitro derivatives has been postulated as: $RNO_2 \xrightarrow{2e} RNO \xrightarrow{2e} RNHOH \xrightarrow{2e} RNH_2$. The intermediate metabolites nitroso and hydroxylamino[9-11] are considered highly toxic reacting with cellular macromolecules leading to necrosis and carcinogenesis.

Nitrazepam was kindly provided by the Industrical Chemical and Pharmaceutical Laboratories, Bombay. $NADP^+$ and glucose-6-phosphate were obtained from Sigma Chemicals, USA. Histidine and biotin of E. Merck (Germany) were used. Agar used was of Difco, USA. Histidine requiring strains of *Salmonella typhimurium* TA 100 and TA 98 were generously provided by Prof. B. N. Ames (Department of Biochemistry, University of Berkeley, USA). The metabolite 2-amino-5-nitrobenzophenone was prepared from nitrazepam (0.035 M) by refluxing for 3 hr in 250 ml ethanolic hydrochloric acid. The solvent was removed under reduced pressure and the reaction mixture cooled. The separated product was crystallized from methanol, m.p. 143–145° C lit[12]. The homogeniety of the product was established by its TLC studies, R_f 0.72 (chloroform-methanol 95:5) and R_f 0.62 (benzene-ethyl acetate 70:30). Its structure was further confirmed through spectral data. IR was characteristic for C = 0 (1639 cm^{-1}, low due to possible intramolecular hydrogen bonding), $-NO_2$ (1540 and 870 cm^{-1}), and —NH (3330 cm^{-1}). ^1H NMR indicated as expected eight aromatic H, δ8.55 (1 Hd, J = 2Hz), 8.35 (1H dd J = 9Hz and 2Hz), δ7.5–7.8 (5 Hm), δ6.85 (1 Hd, J = 9Hz), δ6.9 (NH_2, broad, disappeared after deuterium washing). MS gave molecular ion M^+ 242 and a characteristic fragment at m/e 214 (M-28).

The S-9 fraction routinely employed for microsomal activation was prepared from livers of male rats (Kasauli strain) weighing 200 ± 20 g which had been given an intraperitoneal injection of 500 mg/kg. Aroclor 1254 (Monsanto Co., St. Louis, USA) for the induction of hepatic mono-oxygenases. Five days later, the rats were sacrificed by cervical dislocation. The livers were removed, chopped in a beaker containing 0.15 M KCl, homogenized and centrifuged at 0–4° C in a refrigerated ultracentrifuge at 9000 g as described by Ames *et al.*[1] The supernatant, called S-9 fraction, containing about 40 mg/ml of protein concentration[13] was kept in 2 ml quantities at —80° C in a

freezer. S-9 mixture contained per ml: $Na_2HPO_4.2H_2O$, 100 μmol; $MgCl_2.6H_2O$, 8 μmol; KCl, 33 μmoles; glucose-6-phosphate, 5 μmol; NADP, 4 μmol and 0.4 ml S-9 fraction. The S-9 mixture was filter-sterilized at 4° C using a pressure filtration apparatus fitted with 0.45 μ filter and held at 0–4° C until its use.

The procedure of Ames *et al.*[14] was employed for carrying out mutagenicity testing. Briefly, 0.1–0.2 ml of test sample dissolved in DMSO (spectroscopic grade), 0.1 ml of an overnight bacterial culture (about 10^7 cells) and 0.4 ml S-9 mixture, if required, were added to a test-tube containing 2 ml of molten soft top agar (0.6% agar, 0.3% NaCl, 0.05 mM histidine and 0.05 mM of biotin) kept at 45° C. The ingredients of the tube were mixed and poured onto a petri plate containing 1.5% agar, 2% glucose and minimal inorganic salts. After two days of incubation at 37° C, the histidine revertant colonies were scored. Control plates show only a small number of spontaneous revertant colonies, while plates containing bacteria exposed to the action of mutagen exhibit a larger number of revertant colonies giving a dose-response relationship. Results are expressed as the averages of three replicate plates. Benzo(α)pyrene was used as the positive control.

TABLE 1

Nitrazepam and 2-Amino-5-Nitro Benzophenone and Diagnostic Mutagenic Response in Salmonella typhimurium TA 98 and TA 100

	Dose μg per plate	S-9	Revertants per plate TA 98	TA 100
DMSO control (spontaneous revertants)	0.1 ml	−	30	168
		+	36	181
Nitrazepam	5	−	32	182
		+	37	206
	50	−	50	164
		+	45	150
	100	−	36	175
		+	40	196
	500	−	53	173
		+	56	185
2-Amino-5-Nitro benzophenone	5	−	31	167
		+	45	186
	50	−	52	201
		+	50	207
	100	−	42	164
		+	44	215
	500	−	46	193
		+	50	200
Benzo(α)pyrene	5	−	30	170
		+	—	990

The number of revertant colonies per plate after treatment with the drug and its metabolite is given in table 1. No significant increase in the number of revertant colonies was induced by nitrazepam and its metabolite in the dose range of 5–500 μg/plate. For the results to be significant the number of revertant colonies in the treated plates should be at least twice that of spontaneous revertants. Under similar conditions in the control experiment benzo(α)pyrene in a dose of 5 μg/plate gave 990 as the revertant colonies. The mutagenic metabolite has been demonstrated to be produced when the compound and bacteria were pre-incubated with S-9 mix and then poured on the petri plate as found in N,N-dimethylnitrosamine[15]. Azathioprine requires anaerobic incubation with the bacteria for its mutagenic activity[16]. With these modified techniques nitrazepam and its metabolite showed no significant increase in the number of revertant colonies.

The inability to demonstrate mutagenic effects of nitrazepam, and its metabolite in this sensitive test is significant and encouraging in terms of exposure of human beings to this drug. Literature study also has not revealed any report of tumorogenicity of the drug. Considering the dubious and hazardous character of a number of nitro drugs, nitrazepam seems to be a safer drug for human consumption as far as the genetic toxicity in Salmonella/microsome test of Ames[1] is concerned.

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A NEW ICHNOFOSSIL FROM THE BHANDER GROUP, VINDHYAN SUPERGROUP

S. M. Mathur and N. K. Verma
Centre of Advanced Study in Geology, University of Saugar, Sagar 470 003, India.

It is only in recent years that some of the peculiar markings on sandstones and in limestones of the Vindhyan Supergroup have come to be identified as trace fossils[1-5]. Due to paucity of body fossils in these Precambrian strata, ichnofossils assume considerable importance in furnishing evidence—though indirect—for the existence of Metazoan life. Before they are taken as trace fossils, all markings have to be evaluated carefully to confirm that they were made by animal or plant living at that time, and that they were not the result of any mechanical process of sedimentation.

Records of trace fossils in the Precambrian Vindhyan sedimentary rocks are so rare as to merit documentation, more so when any marking is so unique that it is not reported in existing literature. Such a trace is described in this note.

The slab of a very dusky red-purple quartz-arenite on which the ichnofossil occurs belongs to the Maihar Quartzite formation. It is roughly 13×14 cm in size and is ripple-marked. The morphology of the ripples shows that it is the sole of the layer on which the trace occurs and therefore it is a cast showing convex hyporelief. Since no similar form has been noted in an extensive review of literature, a new ichnogenus *Bhanrerichnus* has been proposed. Systematic description follows.

Ichnogenus Bhanrerichnus Mathur nov.
Ichnospecies *Bhanrerichnus damohensis* Mathur nov.

Figure 1. *Bhanrerichnus damohensis* ichnogen et ichno sp. nov.

Derivation of Name. The generic name is derived from the original spelling of the Bhander Group in which the trace occurs. The specific name refers to the district from which the specimen was collected.

Stratigraphic Horizon. The specimen is a typical example of the Maihar Quartzite of the Bhander Group. In most parts of the basin this formation is the youngest stratigraphic unit of the Vindhyan Supergroup, except in the Bundi area of Rajasthan where another two formations are developed locally above the Maihar Quartzite. Although no direct isotopic date on this formation is available so far, its age is likely to be of the order of 550 Ma on the basis of its position in the stratigraphic column[6].

Locality of the specimen. The specimen was collected from the Sagoni dam site in the Damoh district of Madhya Pradesh. Sagoni (23°49′:79°49′; topo. sheet no. 55M/13) is a railway station on the Bina-Katni section of Central Railway.

Description. The following is the description of the specimen seen from above. Originally the trace must