UNCLASSIFIED
Security Classification

AD 740 981

DOCUMENT CONTROL DATA - R & D

Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author) NAVAL MEDICAL RESEARCH INSTITUTE NATIONAL NAVAL MEDICAL CENTER BETHESDA, MARYLAND 20014	2a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED 2b. GROUP

3. REPORT TITLE

MICROSOMAL METABOLISM OF MORPHINE IN A HYPERBARIC HELIUM ENVIRONMENT

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

Medical research progress report

5. AUTHOR(S) (First name, middle initial, last name)

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6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
AUGUST 1970	4	16

8a. CONTRACT OR GRANT NO b. PROJECT NO. c. d.	9a. ORIGINATOR'S REPORT NUMBER(S) MR005.22-0001, REPORT NO. 1 9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)

10. DISTRIBUTION STATEMENT

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11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
BIOCHEMICAL PHARMACOLOGY, Vol. 20, pp. 2096-2099. Pergamon Press, 1971. Printed in Great Britain	BUREAU OF MEDICINE AND SURGERY (NAVY) WASHINGTON, D. C.

13. ABSTRACT

The effects of hyperbaric helium (21 ata) on the in-vitro microsomal metabolism of morphine was investigated, using liver homogenates from male Sprague-Dawley rats incubated with ^{14}C-morphine at 37°C in specially designed pressure vessels. Pressurized incubations were performed by charging the vessels with a gas mixture of 0.98% oxygen in helium, to a pressure of 294 psig. Control incubations were performed by flushing the vessels with a gas mixture of 80% helium-20% oxygen, at a pressure of 1 ata, but were otherwise identical to pressurized experiments. Morphine and metabolites in the incubation medium were separated by thin layer chromatography after extraction in appropriate solvents, and were quantitated by liquid scintillation counting. These techniques allowed evaluation of both glucuronide formation and N-demethylation of morphine. Under conditions where microsomal enzyme concentration was rate limiting, it was found that there were no differences in glucuronide or normorphine formation between control and pressurized incubations. When substrate concentration was made rate limiting in order to calculate Michaelis-Menton kinetic constants, it was found that hyperbaric helium did not alter the kinetic constants for both reaction.

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14. KEY WORDS	LINK A ROLE	LINK A WT	LINK B ROLE	LINK B WT	LINK C ROLE	LINK C WT
Microsomal Metabolism						
Morphine						
Hyperbaric helium						

ACCESSION for
CFSTI WHITE SECTION ☑
DDC BUFF SECTION ☐
UNANNOUNCED ☐
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Biochemical Pharmacology, Vol. 20, pp. 2096–2099. Pergamon Press, 1971. Printed in Great Britain

Microsomal metabolism of morphine in a hyperbaric helium environment*

(*Received* 6 *August* 1970; *accepted* 16 *October* 1970)

As MAN begins to explore the continental shelf at depths of 600 ft or greater, it is inevitable that his confrontation with the environment will result in conditions demanding immediate treatment with drugs. Treatment will be necessarily administered at depth since safe decompression may take a considerable time period. Because of nitrogen's narcotic effect at depths approaching 250 ft, an atmosphere in which nitrogen is at least partially replaced by helium must be used in deeper saturation diving. A helium-oxygen atmosphere has been shown to support life in man at pressures equivalent to 1500 ft,[1] and in animals to 4000 ft[2] without apparent ill effects. Due to the change in gaseous environment and the possible effects of pressure *per se*, it is conceivable that a drug's interaction with man may be qualitatively and/or quantitatively different at depth than on the surface. The effect of an environment of 20·8 atmospheres absolute (at.a.) of helium with 0·2 at.a. of oxygen on the ability of rat liver microsomes to metabolize morphine was undertaken as an *in vitro* approach to this problem. From this investigation it was concluded that hyperbaric helium does not affect the *in vitro* metabolism of morphine.

Male Sprague-Dawley rats (180–200 g) were decapitated, and their livers were removed and placed in an ice-cold solution of 1·15% KCl-0·02 M tris buffer (pH = 7·4). The livers were blotted, weighed,

* From the Bureau of Medicine and Surgery, Naval Department, Research Task No. MR005.22-9001A. The opinions or assertions contained herein are the private ones of the author and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large. The experiments were conducted according to the principles enunciated in "Guide for Laboratory Animal Facilities and Care".

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and minced, and then homogenized in 2 vol. of KCl-tris solution using a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 9000 *g* for 20 min in a Sorvall refrigerated centrifuge. The supernatant fraction was then centrifuged at 105,000 *g* for 1 hr in a Beckman L-2 ultracentrifuge (fixed angle rotor No. 50). One-half of the supernatant fluid was discarded and replaced by 3 vol. of KCl-tris solution so that in the resuspended mixture 1 ml contained the equivalent of approximately 250 mg of wet liver. The microsomes were used immediately for studies of drug metabolism. Protein was determined according to the method of Lowry *et al.*[3]

A stock solution of radioactive morphine was prepared by dissolving 15 mg of ^{14}C-morphine (9·2 mc/mg, Merck, Sharp and Dohme Laboratories) in 100 ml of water. Fresh morphine solutions were made each day by diluting an aliquot of the stock solution to the appropriate strength with nonradioactive morphine-HCl and KCl-tris solution.

Three ml of incubation medium contained 1·00 mg NAD, 1·32 mg NADP, 10·32 mg isocitrate, 5·08 mg $MgCl_2$ (0·5 ml of 0·05 M solution), 12·00 mg uridine diphosphate glucuronic acid, sodium salt (UDPGA, Sigma Chemical Co.), and 36·33 mg of tris-HCl (2·46 ml of 0·2 M tris buffer, pH = 7·6). All of the biochemicals except UDPGA were obtained from Calbiochem.

Small weighing bottles, each containing 1 ml of microsomal suspension combined with 3 ml of incubation medium, were placed in specially designed pressure vessels[4] in a 37° water bath. The vessels were pressurized to 294 p.s.i.g. (21 at.a.) with a gas mixture of 0·98% oxygen in helium. After equilibration for 8–10 min, 1 ml of the prewarmed radioactive drug solution was injected pneumatically[4] into the vessels, and metabolism was allowed to proceed for a predetermined period of time, after which the vessels were rapidly decompressed and samples were taken for determination of formaldehyde and radioactivity. Control experiments were carried out in exactly the same manner, but the reaction mixture was exposed only to 1 at.a. of a 20% oxygen–80% helium gas mixture. On each day the microsomal suspension prepared from one animal was used to carry out pressurized and control incubations, and each day the order in which pressure or control conditions were imposed was alternated.

From the 5 ml of reaction mixture in each vessel, duplicate 1·8-ml aliquots were used for determination of formaldehyde according to the method of Nash.[5] In addition, duplicate 0·5-ml aliquots were added to separate polyethylene centrifuge tubes, each containing 0·1 ml of 0·1 N NaOH (final pH 8·5), and 8 ml of a mixture of chloroform, isopropanol, and isoamyl alcohol (90:9:1, by vol.). The capped tubes were shaken for 30 min and then centrifuged to separate the two phases. A 0·2 ml aliquot of the aqueous phase was mixed with 0·5 ml of hydroxide of hyamine in a scintillation vial, to which was then added 15 ml of modified Bray's solution (toluene, 1000 ml; dioxane, 1000 ml; methanol, 600 ml; naphthalene, 208 g; PPO, 13 g; POPOP, 0·26 g). Radioactivity was estimated by recording at least 5000 counts per vial with a Packard Tri-Carb spectrometer.

Morphine and metabolites in the aqueous and organic phases were separated by thin layer chromatography (TLC) as described by Yoshimura *et al.*[6] Silica gel, 250 μ thick, was used, with a solvent system of *n*-butanol-acetone-acetic acid-5% ammonium hydroxide-water (45:15:10:10:20, by vol.). To exposed morphine and its metabolites, the TLC plate was sprayed with iodoplatinate solution (10 ml of 10% platinum chloride solution plus 10 g KI, diluted to 500 ml with water).

An apparent dissociation constant (K_m) and the maximal velocity (V_{max}) and their standard errors were obtained by fitting the data to the Michaelis–Menton model* using a nonlinear regression analysis computer program written in BASIC language.[7] This program treated the data in a manner very similar to that described by Davies *et al.*[8] Initial values of K_m and V_{max} were found by using a linear least squares program to fit a straight line to the reciprocal plot of the data (Lineweaver–Burk plot, $1/v$ *v.* $1/s$). These initial values were used as estimates of K_m and V_{max} in the nonlinear regression program which utilized a Taylor series expansion to give an improved estimation of the constants K_m and V_{max}. Differences between pressure and control groups were analyzed by a paired comparison.

In the intact rat the main metabolites of morphine are normorphine and the glucuronides of morphine.[9] The formaldehyde formed during incubation of microsomes with morphine was taken as a measure of *N*-demethylation of morphine to normorphine. The morphine glucuronides formed were determined by extraction of free ^{14}C-morphine from a basic aqueous solution to an organic phase, leaving ^{14}C-morphine glucuronides behind. Corrections were made for unextracted morphine and formaldehyde, and for background. A time course study is summarized in Table 1. At no time was there a significant difference between pressure and control groups in either glucuronide formation or *N*-demethylation.

Six concentrations of morphine ranging from 0·1 to 2·0 mM were used to determine kinetic constants for morphine metabolism under pressure and control conditions. An incubation time of 7 min was used in these experiments. The apparent K_m and the apparent V_{max} for both the *N*-demethylating

* $v = (s)(V)/(s + K)$, where s = substrate concentration and v = rate of reaction.

TABLE 1. EFFECT OF HYPERBARIC HELIUM ON THE METABOLISM OF MORPHINE

Minutes of incubation	*N*-Demethylation			Glucuronide formation		
	Control value*	Effect on metabolism†	P‡	Control value*	Effect on metabolism†	P‡
1	0·52±0·12	0·03	> 0·4	0·64±0·16	0·16	> 0·05
2	1·00±0·21	−0·04	> 0·1	0·76±0·21	−0·19	> 0·1
3	1·34±0·18	0·01	> 0·5	1·39±0·22	−0·01	> 0·5
5	2·28±0·43	−0·03	> 0·5	2·32±0·29	0·06	> 0·5
7	2·89±0·47	0·04	> 0·05	2·73±0·46	−0·16	> 0·5
9	3·58±0·51	0·01	> 0·5	3·47±0·39	0·13	> 0·05

* Means ± S.E. (m-μmoles/mg protein), $n = 6$.
† Difference in metabolism (pressurized *minus* control; m-μmoles/mg protein) for each experiment. Values represent mean differences of six experiments.
‡ P refers to a paired comparison between pressurized and nonpressurized groups.

TABLE 2. EFFECT OF HYPERBARIC HELIUM ON THE KINETIC CONSTANTS OF MORPHINE METABOLISM*

Metabolic pathway	K_m†			V_{max}‡		
	Pressure (21 at.a.)	Control (1 at.a.)	P§	Pressure (21 at.a.)	Control (1 at.a.)	P‡
N-Demethylation	0·54±0·04	0·54±0·04	> 0·5	4·36±0·21	4·46±0·25	> 0·5
Glucuronide formation	0·25±0·02	0·23±0·02	> 0·2	3·64±0·35	3·57±0·36	> 0·2

* Incubation time used for this assay = 7 min.
† Mean (mM) ± S.E., $n = 9$.
‡ Mean (m-μmoles/mg protein/7 min) ± S.E., $n = 9$.
§ P refers to a paired comparison between pressurized and control groups.

system and the glucuronyl transferase system are shown in Table 2. There were no significant differences between pressure and control kinetics.

In previous work, hyperbaric helium was found to stimulate the rate of oxidation of hexobarbital by rat liver microsomes during the first 10 min of incubation.* The NADPH-requiring aliphatic hydroxylation system that metabolizes hexobarbital is probably closely associated with the *N*-demethylase system, but may be more discrete from the glucuronide forming system.[10] Nevertheless the present study did not reveal any effects of a hyperbaric atmosphere on the initial rate of metabolism of morphine or on the kinetic constants, K_m and V_{max}, associated with the *N*-demethylase system or the glucuronyl transferases. Other evidence of an effect of helium on oxidative processes was reported by Cook,[11] who demonstrated that helium at 1 at a increased the rate of glycolysis in mouse liver homogenates undergoing oxidative metabolism. One at.a. of helium increased the rate of oxygen consumption and decreased the development time of *Drosophila* and *Tenebrio*, and also increased the rate of aerobic metabolism of various reptiles and whole mice.[11] Conversely, the rate of anaerobic metabolism in mouse liver slices was decreased by 1 at.a. of helium.[12] Helium at 150 at.a. inhibited the growth rate of Hela cells in monolayer cultures,[13] and at 30 at.a. depressed the growth rate of the mold, *Neurosporo crassa*.[14]

The results of the present investigation are in agreement with others who have found that helium does not affect drug action. The toxicity of several drugs was not altered in rats and mice exposed to 19·2 at.a. of helium,[15] and there were no significant effects on the electrocardiogram and on the toxicity

* G. K. HANANEDNO and S. I. SCHWARTZ, unpublished observations.

of cardiac glycosides in cats or rats exposed to 19·2 ata of helium.* However, other pharmacological aspects of drug action may be influenced by a hyperbaric environment. Indeed, hyperbaric helium (69 at.a.) enhanced the resistance of several strains of *S. aureus* to penicillin.[16] In addition, Small *et al.** found that the chronotropic response to ouabain and digoxin was slightly altered in guinea pigs exposed to 19·2 at.a. of helium. Moreover, prolonged exposure of men to a hyperbaric helium environment may result in enzyme induction or a decrease in the amount of enzyme. Thus, drug metabolism might be altered under such conditions even though acute exposure, as shown in the present *in vitro* experiments, does not alter activity or kinetics of selected enzyme systems. Therefore, additional research is necessary to ascertain that hyperbaric conditions do not affect the actions of drugs that might be used in an underwater habitat.

Acknowledgements—The authors wish to thank Mrs. Linda Martinez for her excellent technical assistance, and gratefully acknowledge the gift of ^{14}C-morphine by Merck, Sharp and Dohme Laboratories, Rahway, N.J.

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