mers (0.84 mM), and in each case a 30 M excess of (S,S)-IV or (S)-I. The reactions were performed in screw cap sealed, polytef-lined reaction vials which were heated to 75–100° for 1–4 hr in a block heater. At the end of the reaction, the mixtures were washed with 0.2% NaOH (50 μl) followed by 0.5% HCl (50 μl) and then dried over sodium sulfate. The solutions were carefully transferred with the aid of a Pasteur pipet to a second reaction vial and the solvent removed under a stream of dry nitrogen. The residues were dissolved in 100 μl of toluene, and 1 μl of the resulting solution was analyzed by GC. Retention times for (S,S)-XIV and (S,R)/(R,S)-XV were 16.8 and 19.2 min, respectively. Derivatization of (S)-α-methylbenzylamine with (S,S)-IV and (R,S)-V proceeded in a similar fashion. GC retention times for (S,S)-VII and (S,R)-VIII were 15.5 and 13.3 min, respectively, using the same conditions as for the ketamine analyses.

REFERENCES

(1) N. P. McGraw, P. S. Callery, and N. Castagnoli, Jr., *J. Med. Chem.*, **20**, 185 (1977).

(2) L. K. Low and N. Castagnoli, Jr., "Annual Reports in Medicinal Chemistry," vol. 13, F. H. Clarke, Ed., Academic, New York, N.Y., 1978, pp. 304–315.

(3) E. Gil-Av and D. Nurok, *Adv. Chromatogr.*, **16**, 99 (1978).

(4) I. Tomida and M. Matsuzaki, *Agr. Biol. Chem.*, **43**, 925 (1979).

(5) S. B. Matin, M. Rowland, and N. Castagnoli, Jr., *J. Pharm. Sci.*, **62**, 821 (1973).

(6) J. D. Adams, N. Castagnoli, and A. J. Trevor, *Proc. West. Pharmacol. Soc.*, **21**, 471 (1978).

(7) F. Weygand, P. Klinke, and I. Eigen, *Chem. Ber.*, **90**, 1896 (1957).

(8) I. Tomida and T. Kuwahara, *Agr. Biol. Chem.*, **42**, 1059 (1978).

(9) H. D. W. Hill, A. P. Zens, and J. Jacobus, *J. Am. Chem. Soc.*, **101**, 7090 (1979).

(10) S. Wolfe and P. M. Kazmaier, *Can. J. Chem.*, **57**, 2388 (1979).

(11) M. W. Williams and G. T. Young, *J. Chem. Soc.*, **4**, 3701 (1964).

(12) M. Goodman and K. C. Steubern, *J. Org. Chem.*, **27**, 3409 (1962).

(13) Bristol Myers Co., British pat. 1 330 878 (1973).

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health training grants GM 23918-01 and GM 07175-04, and research grants GM 01791 and NS 17956.

Thomas Woolf is an AFPE H. A. B. Dunning Memorial Fellow.

In Vivo and *In Vitro* Studies with Sulfamate Sweeteners

GRAINNE McGLINCHEY, C. BERNADETTE COAKLEY, VIDA GESTAUTUS-TANSEY, JOHN GAULT *, and WILLIAM J. SPILLANE [x]

Received August 28, 1981, from the *Chemistry Department, University College, Galway, Ireland.* Accepted for publication, September 28, 1981. *Present address: Regional Technical College, Sligo, Ireland.

Abstract ☐ The sweet compounds 2-methyl- and 3-methylcyclohexyl- and 2-cyclohexenylsulfamates were fed to Wistar albino rats. The urine (and feces in the case of 2-cyclohexenylsulfamate) was examined for possible amine, ketone, and alcohol metabolites. The total percent of metabolites formed was low and the hexenyl compound gave a particularly small quantity of metabolite. The results with these compounds are compared with those obtained from earlier *in vivo* studies with cyclamate and other sulfamates. In complementary *in vitro* studies, the four sweetest sulfamates, namely, cyclamate, cycloheptyl-, cyclooctyl-, and cyclopentylsulfamates were incubated with the cell-free extract of bacteria isolated from the feces of cyclamate fed rats. Some correlation was apparent between these *in vitro* experiments and previous *in vivo* studies. Preliminary mutagenicity testing (the Ames test) of some amines (corresponding to the sulfamates studied) has been carried out.

Keyphrases ☐ Sulfamate sweeteners—*in vivo* and *in vitro* studies of amine, ketone, and alcohol metabolites ☐ Metabolism—amine ketone, and alcohol metabolites of sulfamate sweeteners ☐ Cyclamates—*in vivo* and *in vitro* studies of amine, ketone, and alcohol metabolites

The controversial ban on the use of cyclohexylsulfamate (cyclamate) salts as nonnutritive sweeteners has provided a strong impetus for wide and varied toxicity studies of these compounds and their metabolites (1). Though at least some other sulfamates have a sweetness potency similar to the banned parent compound (2), and it has been suggested that certain of these sulfamates might be less readily metabolized than is cyclamate (3), nevertheless toxicological studies have been carried out on just a few such compounds (4). In the present work, *in vivo* animal feeding studies employed three other sweet sulfamates: 2- and 3-methylcyclohexyl- and 2-cyclohexenylsulfamates. In a complementary *in vitro* study, some of the sweetest known sulfamates have been incubated in cell-free extracts of the microorganisms responsible for the metabolism of cyclamate. Some work has been carried out on the mutagenicity of the amines to which these sulfamates give rise when metabolized.

EXPERIMENTAL

Reagents and Chemicals—The amines, ketones, and alcohols were commercially available and were used as obtained. The following compounds were synthesized by known literature methods: 2-methylcyclohexanol (5), cyclohexyl- (6), 2-methyl- (6), 3-methyl- (6), 2-cyclohexenyl- (7), n-octyl- (8), and phenyl- (9) sulfamates. Cyclopentyl-, cycloheptyl-, and cyclooctylsulfamates were previously prepared (4). All the synthesized sulfamates (as their sodium salts) gave a positive "sulfamate test" (2), satisfactory C, H, and N analysis, and the characteristic IR bands for sulfamates. 2-Cyclohexenylsulfamate gave an additional band in the 1640–1615 cm^{-1} region, which is characteristic of an ethylenic double bond.

***In Vivo* Experiments**—Female Wistar albino rats (200–257 g) were kept on solid food and water in rat metabolism cages[1]. The sodium salts of 2- and 3-methylcyclohexyl- and 2-cyclohexenylsulfamates were administered orally in water (~20 ml) at a level of 1.45 g/kg of body weight to groups of six rats (five in the case of 2-methylcyclohexylsulfamate). Prior to administration of the sulfamates, the rats were deprived of water for 24 hr. Prior to feeding, the urine of each rat was examined for metabolites by GLC. Similarly, the three sulfamates fed were screened for occluded amine, also by GLC. After administration of each sweet compound, the urine (and feces in the case of 2-cyclohexenylsulfamate) was collected for 3 days, bulked, and refrigerated for no more than 1 day when GLC analysis for the metabolites was carried out.

The urine and feces recovered from rats, fed 2-cyclohexenylsulfamate, was not analysed until the eighth day (instead of the customary fourth day), and accordingly, a study of the stabilities of 2-cyclohexenylamine and 2-cyclohexenone in urine and feces over a 7-day period was made.

[1] NKP, Kent, England.

0022-3549/82/0600-0661$01.00/0

© 1982, American Pharmaceutical Association

Table I—Percent Recovery of 2-Methylcyclohexylamine, 2-Methylcyclohexanone, and 2-Methylcyclohexanol from Urine

Amine		Ketone		Alcohol	
μg	%	μg	%	μg	%
0.015	68.7	0.02	88.0	0.028	81.5
0.03	83.0	0.05	90.3	0.056	86.0
0.10	100.5	0.10	95.6	0.08	91.0
0.10	99.0	0.10	96.3	0.10	98.5
0.15	86.3	0.15	87.0	0.10	96.3
0.2	93.5	0.20	91.5	0.14	89.3
0.25	95.6	0.20	93.0	0.17	85.1
0.25	98.5	0.25	99.0	0.17	97.3
Mean	90.64		92.58		90.63
±*SE*	±8.41		±3.38		±5.15

The two metabolites were added individually to samples of rat urine and feces, and these samples were then refrigerated for 7 days, worked-up, and analyzed in the same manner as the actual samples from the feeding experiments.

General Work-Up Procedure, Standardization, and GLC Analysis—The details for other sulfamates have been reported previously (4). Analogous procedures and methods were employed for the three sulfamates. The GLC conditions were as follows. 2-Methylcyclohexylsulfamate metabolites: column temperature, 117°; gas flow rates, nitrogen, 60 ml/min, hydrogen, 60 ml/min, and overall (including air), 500 ml/min; retention times (min), 2-methylcyclohexylamine (4.5), 2-methylcyclohexanone (6.7), 2-methylcyclohexanol (10.1), and 4-methylcyclohexanol (internal standard) (12.6). 3-Methylcyclohexylsulfamate metabolites: column temperature and flow rates, as described previously; retention times (min), *n*-undecane (internal standard) (3.8), 3-methylcyclohexylamine (4.6), 3-methylcyclohexanone (7.2), and *cis*- and *trans*-3-methylcyclohexanols (10.5 and 11.7, respectively). 2-Cyclohexenylsulfamate metabolites: column temperature, 107°; gas flow rates were identical with those described previously; retention times (min), *n*-dodecane (internal standard) (11.0), 2-cyclohexenone (16.5), and 2-cyclohexenylamine (18.6). The lengthy retention times involved in the latter analysis could not be shortened on the column (4). This is because a rise in the temperature tended to cause the cyclohexenyl metabolite peaks to merge. The GLC conditions for analysis of the sulfamate metabolites used in the *in vitro* experiments have been described previously (4), except in the case of *n*-octyl- and phenylsulfamates. The following are the GLC conditions for the determination of the metabolites of these two sulfamates. *n*-Octylsulfamate metabolites: column temperature, 130°; nitrogen gas flow rate, 150 ml/min; retention times (min); *n*-octylamine (6.4), tridecane (internal standard) (9.6), and *n*-octanol (19.5). Phenylsulfamate metabolites: column temperature and nitrogen flow rate as for *n*-octylmetabolites; retention times (min), aniline (3.8), tridecane (internal standard) (9.6), and phenol (31.0).

***In Vitro* Experiments**—*Isolation of Organisms and Preparation of Cell-Free Extracts*—Wistar albino rats were fed cyclohexylsulfamate at the rate of 2 g/week for a 10-week period. The urine was tested for the presence of cyclamate metabolites, *i.e.*, cyclohexylamine, cyclohexanone, and cyclohexanol, using solvent extraction and identification by GLC (10). When a rat was consistently breaking down cyclamate to its metabolites, the feces were then used for the isolation of the microorganisms.

A nutrient broth agar containing 1% cyclohexylsulfamate was inoculated with a sample of fecal material, allowed to grow overnight, and transferred to a larger volume the following day. The bacteria were grown in a shaking incubator for 5 days after which they were harvested by centrifugation. The pellets were resuspended in a small volume of tromethamine-hydrochloride buffer, pH 7.2, and lysed by sonication.

Incubation Procedure and Analysis for Metabolites—Two milliliters of cell-free extract were incubated with 200 μmoles of test sulfamate in 2 ml of 0.1 *M* phosphate buffer, pH 6.8, for 2 hr at 50°. The reaction was stopped by adding 1 ml of 10 *N* NaOH followed by 4 ml of 20% sulfosalicylic acid.

The incubation mixture was extracted three times with 20-ml volumes of methylene chloride. The methylene chloride extracts were bulked and evaporated to dryness and the residue reconstituted with 1 ml of methylene chloride. Analysis for metabolites was then carried out using the procedure described previously. The incubation studies were carried out over a 2-hr period at 50°, pH 6.8, and were the conditions under which the enzymes present in the cell-free extract displayed maximum activity. The variations of enzyme activities with temperature, pH, and incubation time were determined in separate experiments.

Mutagenicity Testing—Amines were tested for their ability to cause base-pair substitution mutations using the mutant strain of *Salmonella typhimurium* TA 1535, according to the method described previously (11). Fifteen micrograms of the test substance contained in 0.1 ml of water was used in each case and 2-aminofluorene was used as a standard.

The mixed oxidase S-9 mixture was not used because the compounds under test were already presumed metabolites.

Table II—Percent Recovery of 3-Methylcyclohexylamine, 3-Methylcyclohexanone, and 3-Methylcyclohexanol from Urine

Amine		Ketone		Alcohol	
μg	%	μg	%	μg	%
0.05	73.5	0.01	105.7	0.05	75.6
0.15	85.6	0.02	78.6	0.15	89.1
0.25	105.3	0.03	79.8	0.25	93.0
0.25	96.5	0.10	98.6	0.40	98.0
0.45	103.0	0.10	102.5	0.40	89.1
0.70	98.4	0.20	97.8	0.60	103.5
1.20	99.0	0.30	95.4	0.80	98.2
Mean	94.47		94.06		92.36
±*SE*	±8.53		±8.49		±6.65

RESULTS AND DISCUSSION

In the present paper the three compounds, 2-methyl- and 3-methylcyclohexyl- and 2-cyclohexenylsulfamates, were administered as their sodium salts to rats. These studies extended previous studies in which sulfamates other than the "parent" sweet sulfamate, cyclohexylsulfamate (cyclamate), were fed to animals and the urine examined for breakdown products (4). Prior to administration of the three compounds in the present study, standard curves and percent recoveries of likely metabolites were determined using procedures established in earlier work (4). In Tables I–III, percent recoveries for metabolites of 2-methyl- and 3-methylcyclohexyl- and 2-cyclohexenylsulfamates are seen to be satisfactory. It should be noted that in general the quantities in micrograms used in the determination of the percent recoveries are in the same range as the amounts of metabolites found in the feeding experiments.

In other preliminary experiments, the sulfamates given to the animals were screened for small occluded amounts of metabolite(s) and the urine (feces in one instance) of the rats to be fed was screened for metabolites. Since it was not possible to examine the urine/feces of the rats that were fed 2-cyclohexenylsulfamate for 7 days after administering the sweetener, a study of the stabilities of 2-cyclohexenylamine and 2-cyclohexenone in urine/feces was made over a 7-day period. The stabilities of the two compounds, reported as percent survival, is excellent (Table IV). Samples of 2-cyclohexenols were not available but it appears unlikely that they were products of the metabolic cleavage of 2-cyclohexenylsulfamate.

The aim of the *in vivo* study was to examine the effect of introducing a substituent or double bond on metabolic breakdown of cyclohexylsulfamate. If metabolic breakdown was decreased, then the risks associated with cyclamate ingestion should be reduced. The first compound administered was 2-methylcyclohexylsulfamate. Table V shows that this compound was broken down by all rats to the metabolites 2-methylcyclohexylamine and/or 2-methylcyclohexanone. None of the rats in this group gave alcohol metabolites. In this work, as previously (4), the percent conversion to a particular metabolite is defined as the amount of metabolite found (milligrams) divided by the amount of sulfamate fed (milligrams) multiplied by a hundred times the ratio of the molecular weight of the sulfamate to that of the particular metabolite. In this instance, the mean percent conversions to amine and ketone are 0.00013 and 0.00067, respectively. In the case of the study with 3-methylcyclohexylsulfamate, the mean percent of 3-methylcyclohexylamine, 3-methylcyclohexanone, and 3-methylcyclohexanol formed were 0.014, 0.0002, and 0.0098, respectively (Table VI). After administration of 2-cyclohexenylsulfamate, only two rats in the group of six were found to be converters, and interestingly, these two rats gave amine only (mean percent, 0.00017) with no metabolites in their feces (Table VII). It was not possible to check positively for the presence of 2-cyclohexenols, since these compounds were not available. However, from experience it was felt that the retention time(s) of this alcohol(s) should be fairly close to that of the corresponding amine and ketone, but no traces were obtained in the vicinities of the peaks due to these metabolites, nor did any traces appear when the chromatograph was run 29 min, *i.e.*, 10 min after the appearance of the last peak.

Table VIII gives the mean percent and the percent total metabolites found in *in vivo* studies with the three sulfamates of the present study, five sulfamates previously administered to rats and, for comparison, some typical data for cyclohexylsulfamate.

Table III—Percent Recovery of 2-Cyclohexenylamine and 2-Cyclohexenone from Urine and Feces

Urine				Feces			
Amine		Ketone		Amine		Ketone	
µg	%	µg	%	µg	%	µg	%
1.5	101.7	2.0	97.9	1.5	76.2	2.00	60.5
4.0	139.0	3.0	73.1	4.0	93.9	5.00	68.5
6.0	99.3	4.0	103.0	6.0	99.2	7.5	71.3
6.0	105.2	6.0	86.5	7.0	93.7	8.5	86.2
7.0	98.3	10.0	104.2	8.0	106.4	9.5	105.3
8.0	100.8			8.0	97.6	10.0	85.4
Mean	107.4		92.94		94.5		79.5
±*SE*	±10.55		±10.5		±6.35		±12.77

Table IV—Stability of 2-Cyclohexenylamine and 2-Cyclohexenone in Urine and Feces over a 7-Day Period

Urine				Feces			
Amine		Ketone		Amine		Ketone	
Sample Added, µg	Survival, %	Sample Added, µg	Survival, %	Sample Added, µg	Survival, %	Sample Added, µg	Survival, %
5.8	99.0	7.2	83.5	5.8	92.5	7.2	71.5
5.8	96.5	7.2	90.2	5.8	86.4	7.2	85.2
5.8	90.3	7.2	104.3	5.8	98.2	7.2	87.2
Mean ± *SE*	95.3 ± 3.3		92.7 ± 7.8		92.4 ± 4.0		81.3 ± 6.5

Table V—Metabolism of 2-Methylcyclohexylsulfamate in Rats

	Metabolites						
	2-Methylcyclohexylamine		2-Methylcyclohexanone		2-Methylcyclohexanol		
Animal Number	mg	%	mg	%	mg	%	% Total Metabolites
1	0.0016	0.00012	0.00012	0.00009	None	0	0.00021
2	0.00049	0.00037	None	0	None	0	0.00037
3	None	0	0.0031	0.0024	None	0	0.00024
4	0.00013	0.0001	0.0005	0.00036	None	0	0.00046
5[a]	0.00007	0.00007	0.0004	0.00048	None	0	0.00055
Mean	0.00017	0.00013	0.00082	0.00067			0.00037
±*SE*	±0.00013	±0.00009	±0.0009	±0.00069			±0.00011

[a] Fed only 0.17 g instead of 0.26 g, based on 1.45 g/kg body weight.

Table VI—Metabolism of 3-Methylcyclohexylsulfamate in Rats

	Metabolites						
	3-Methylcyclohexylamine		3-Methylcyclohexanone		3-Methylcyclohexanol		
Animal Number	mg	%	mg	%	mg	%	% Total Metabolites
1[a]	0.013	0.014	None	0	0.026	0.028	0.042
2	0.01	0.008	None	0	None	0	0.008
3	0.062	0.03	None	0	0.049	0.027	0.057
4	0.066	0.03	None	0	None	0	0.030
5	None	0	0.00246	0.0012	0.008	0.004	0.0052
6	0.006	0.003	None	0	None	0	0.003
Mean	0.026	0.014	0.00041	0.0002	0.0138	0.0098	0.0242
±*SE*	±0.025	±0.011			±0.0158	±0.012	±0.0188

[a] Rat 1 received approximately half the amount of sulfamate.

Table VIII shows that the degree of metabolic cleavage by rats of various types of sulfamates is low, being generally <0.1%. Since the number of rats involved in each of the feeding studies was small, *i.e.*, five or six, any deductions made from the results in Table VIII regarding the relative stability of sulfamates would be tenuous. However, it seems that cyclooctylsulfamate, which gives rise to all three metabolites, is cleaved to a somewhat greater extent than the other sulfamates. On the other hand, the introduction of a double bond into the cyclohexyl system, *i.e.*, in 2-cyclohexenylsulfamate, appears to reduce metabolic breakdown considerably. This latter compound has a comparable level of sweetness to cyclamate (7). Interestingly, when the closely related and sweet 3-cyclohexenylsulfamate was administered intraduodenally for 2 weeks to rats at a dosage of 1 g/kg of body weight, no pathological changes in the main organs or tissues could be detected (7).

One of the sulfamates in Table VIII is not sweet; however, on the basis of a previous cursory examination, it was taken to be sweet (4). On careful examination it was classified as being bitter (13). Unterhalt and Böschemeyer (14), who first made this compound, also reported it as being nonsweet.

Complementary to the *in vivo* studies, a series of *in vitro* experiments have been carried out. In this study, the microorganisms responsible for the metabolism of cyclamate were isolated from the feces of rats fed cyclamate. A cell-free extract of these organisms was then incubated with the four sweetest known sulfamates and two standards to ascertain the extent to which these compounds were broken down to potentially toxic metabolites.

Earlier work (15) showed that dogs and humans fed on cyclamate

Table VII—Metabolism of 2-Cyclohexenylsulfamate in Rats

	Metabolites			
	2-Cyclohexenylamine		2-Cyclohexenone	
Animal Number	mg	%	mg	%
1	None	0	None	0
2	0.00047	0.00036	Trace	0
2[a]	Trace	0	Trace	0
3	None	0	None	0
4	0.00083	0.00068	Trace	0
4[a]	Trace	0	Trace	0
5	None	0	None	0
6	None	0	None	0
Mean	0.00022	0.00017[b]		
±*SE*	±0.00028	±0.00023		

[a] Results for feces. [b] The mean was obtained by dividing the summation of the percent of metabolite in the urine by 6.

Table VIII—Metabolism of Sulfamates in Rats

Cyclic Sulfamate	Percent Metabolites[a]			Total Metabolites, %	Reference
	Amine	Ketone	Alcohol		
Octyl	0.127	0.08	0.092	0.297	4
Heptyl	0.0514	0.0088	0.0034	0.064	2[b]
Hexyl[c]	0.0096	0.00095	0.004	0.0146	12
2-Methylhexyl	0.00013	0.00067	0	0.00037	This work
3-Methylhexyl	0.014	0.0002	0.0098	0.0242	This work
4-Methylhexyl	0.0073	0.0013	0	0.0085	4
2-Hexenyl	0.00017	0	0	0.00017	This work
Pentyl	0.057	0.016	0.0087	0.083	1[d]
Pentylmethyl	0.007	—[e]	0.007	0.0144	3[f]

[a] These percents were calculated by summing the percent metabolite for each rat, summing the totals, and dividing by the number of rats in each study. [b] Reference 2 in (4). [c] The figures for hexyl were obtained from data on six rats (2 groups of 3). The percent total metabolites represents the total for amine, ketone, and alcohol. In Ref. 12 the authors also detected "conjugated" cyclohexanol as a metabolite, so their total differs slightly. [d] Reference 1 in (4). [e] A ketone metabolite is not possible for this compound. [f] Reference 3 in (4).

produced large quantities of a metabolite, cyclohexylamine, in their urine. It was considered that this compound was the carcinogen, and it seemed likely that the conversion from cyclohexylsulfamate to cyclohexylamine was carried out by processing enzymes in the liver. Attempts to verify this *in vitro* study, however, met with little success (16).

The formation of cyclohexylamine and cyclohexanone from cyclamate by microorganisms isolated from guinea pig feces was demonstrated previously (17). This work was followed up by carrying out a partial purification of the enzyme involved, cyclohexylsulfamatase (18). The substrate specificity of the partially purified enzyme using several other sulfamates, which were generally not sweet, was also examined.

These studies subsequently partially purified and examined the properties of cyclohexylamine oxidase, which is the next enzyme in the metabolic sequence (19):

Biochemical tests for Gram-negative enteric bacteria were first carried out on the bacteria whose cell-free extract carried out the sulfamatase function. These tests included indole production; hydrogen sulfide production; liquefaction of gelatin; cleavage of sugars, alcohols, and glucosides; and methyl red Voges Proskauer tests. The results of these tests indicated the presence of *Escherichia coli*, *Enterobacter hafniae*, and *Proteus mirabilis*. Pure cultures of these organisms were grown and cell-free extracts incubated with cyclohexylsulfamate, but it was found that greater activity was observed when a mixed culture was used.

A series of investigations was carried out to determine the optimum conditions for assay of enzyme activity. The variation in crude enzyme activity in relation to temperature, pH, and time of incubation was investigated and as a result, it was decided to adopt the incubation conditions as outlined previously: pH 6.8 for 2 hr at 50°.

Partial purification of the enzyme by ammonium sulfate fractionation (18) resulted in greatly reduced activity. Therefore, it was decided to conduct the studies using the crude enzyme extract. It has also been reported (18) that heat treatment of the crude enzyme extract (60° for 10 min) inactivates heat labile enzymes which are responsible for further degradation of cyclohexylamine, while leaving the heat stable sulfamatase unaffected. However, experience showed that the sulfamatase itself is largely inactivated by this procedure so it was not adopted. Consequently, the crude enzyme extract was used in the incubation studies.

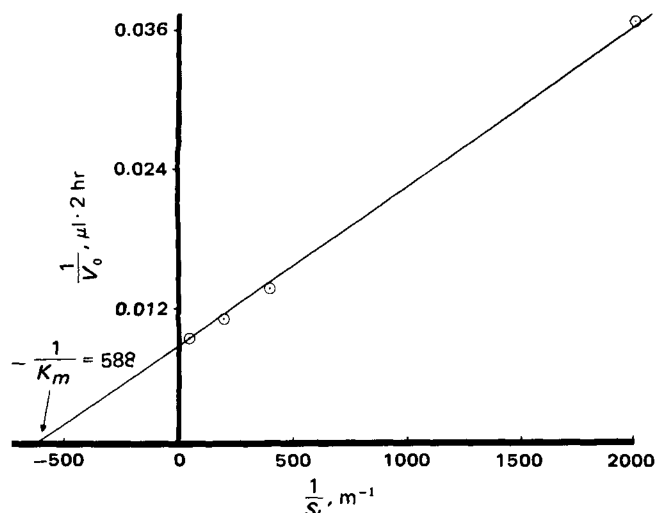

Figure 1—*Lineweaver-Burk plot for cyclohexylsulfamate.*

Table IX—Sulfamate Specificity of the Enzyme Extract and Mutagenicity (Ames Test) of the Corresponding Amines

N-Substituted Sulfamate	Relative Activity	Relative Sweetness[b]	Mutagenicity of Parent Amine[c]
Cyclohexyl	1[a]	1	0.02
Phenyl	5.3	—	—[d]
n-Octyl	6.4	—	—[d]
Cycloheptyl	0.52	0.83	—[d]
Cyclopentyl	0.50	0.24	0.006
Cyclooctyl	0	0.67	—[d]

[a] Total breakdown was 0.5%. [b] Data taken from Ref. 2; no values indicate not sweet. [c] Compared to standard 2-aminofluorene which is equal to one. [d] Zero or negligible.

In Table IX the results of incubation of various sulfamates with this enzyme extract are shown relative to cyclohexylsulfamate. The activities are computed as the sum of metabolites, *i.e.*, amine, alcohol, and ketone. Since it was found that *n*-octylsulfamate (which is not sweet) was the most labile substrate examined (18), this compound was included in the present study to assess the activity of the enzyme extract. As can be seen from Table IX, the enzyme extract was also highly active against this substrate, giving a relative activity of 6.4 as compared to cyclohexylsulfamate.

Phenylsulfamate was also included as another standard and it was found that it too was quite labile, in contrast to previous results, which indicated that the enzyme had only a very weak or uncertain activity on this substrate. It was confirmed that this breakdown was not due to impurities in the sulfamate and/or nonenzymatic hydrolysis. This was done by incubating the buffered substrate in the absence of the enzyme extract when no aniline or phenol was detected.

A variety of sulfamates was incubated previously (18), but apart from cyclohexylsulfamate, only two faintly sweet sulfamates, *n*-propyl- (2) and *ac*-tetrahydro-β-naphthyl (6) sulfamates, were included in the study. For the current incubation experiments, the four sweetest known sulfamates, cyclohexyl-, cycloheptyl-, cyclooctyl-, and cyclopentyl-, were incubated. Relative to cyclohexylsulfamate, all three had less activity. There was a partial correlation between the *in vivo* results in Table VIII for these four compounds and the *in vitro* results for the sulfamates in Table IX. Thus, in both types of experiments the heptyl- and pentyl-compounds were metabolized ~50% that of cyclohexylsulfamate. In the case of cyclooctylsulfamate, the results from the *in vivo* and *in vitro* studies contradict each other. The Michaelis constant (Km) for the enzyme with cyclohexylsulfamate was determined using a Lineweaver-Burk plot (Fig. 1) and was found to be 1.7×10^{-3} *M*. This indicates a three times greater affinity between enzyme and substrate than that reported previously (18).

In Table IX, preliminary studies on the mutagenicity of the amines of the sulfamates which were incubated are reported. The amines of all the sulfamates reported in Table IX show zero or negligible mutagenic activity [in the case of aniline this result was already established (20)]. The amines, isobutylamine and isoamylamine, both of whose sulfamates are moderately sweet (compared to cyclamate) (2), also show negligible mutagenicity, but *n*-butylamine, whose sulfamate is faintly sweet (2),

appeared to be mutagenic (60 revertants/15 μg). It should be stressed, however, that these are preliminary studies and would have to be confirmed using other tester strains of *Salmonella typhimurium*.

REFERENCES

(1) G. McGlinchey, Ph.D. thesis, National University of Ireland, 1980.
(2) G. A. Benson and W. J. Spillane, *J. Med. Chem.*, **19**, 869 (1976).
(3) W. J. Spillane, *J. Pharm. Sci.*, **62**, 1394 (1973).
(4) W. J. Spillane, G. A. Benson, and G. McGlinchey, *ibid.*, **68**, 372 (1979).
(5) "Organic Synthesis," coll. Vol. 2, A. H. Blatt, Ed., Wiley, New York, N.Y., 1966, p. 317.
(6) L. F. Audrieth and M. Sveda, *J. Org. Chem.*, **9**, 89 (1944).
(7) M. Avramoff and W. Taub, *Eur. J. Med. Chem.*, **13**, 81 (1978).
(8) C. Nofre and F. Pautet, *Bull. Soc. Chim. Fr.*, **1975**, 686.
(9) P. Baumgarten, *Chem. Ber.*, **59**, 1166 (1926).
(10) G. A. Benson and W. J. Spillane, *J. Chromatogr.*, **136**, 318 (1977).
(11) B. N. Ames, J. McCann, and E. Yamasaki, *Mutation Res.*, **31**, 347 (1975).
(12) A. Suenaga, S. Kojima, and H. Ichibagase, *ibid.*, **20**, 1357 (1972).
(13) W. J. Spillane and G. McGlinchey, *J. Pharm. Sci.*, **70**, 933 (1981).
(14) B. Unterhalt and L. Böschemeyer, *Z. Lebensm.-Unters.-Forsch.*, **145**, 93 (1971).
(15) S. Kojima and H. Ichibagase, *Chem. Pharm. Bull.*, **14**, 971 (1966).
(16) B. S. Drasar, A. G. Renwick, and R. T. Williams, *Biochem. J.* **129**, 881 (1972).
(17) M. Asahina, T. Niimura, T. Yamaha, and T. Takahashi, *Agr. Biol. Chem.*, **36**, 711 (1972).
(18) T. Niimura, T. Tokieda, and T. Yamaha, *J. Biochem. (Japan)*, **75**, 407 (1974).
(19) T. Tokieda, T. Niimura, F. Takamura, and T. Yamaha, *ibid.*, **81**, 851 (1977).
(20) J. McCann, E. Choi, E. Yamasaki, and B. N. Ames, *Proc. Natl. Acad. Sci. USA*, **72**, 5135 (1975).

ACKNOWLEDGMENTS

The authors thank the National Board for Science and Technology (Ireland) for a research grant, P. Considine (Galway) and E. Brady (Sligo) for their assistance with microbiological aspects of this work, and Professor B. Ames (Berkeley) for a sample of *Salmonella typhimurium* TA1535.

Plasma Levels of a Novel Antidysrhythmic Agent, Meobentine Sulfate, in Humans as Determined by Radioimmunoassay

JAMES T. WARREN *, GEOFFREY G. COKER ‡, RICHARD M. WELCH *, ARTHUR S. E. FOWLE ‡, and JOHN W. A. FINDLAY *x

Received July 27, 1981, from the * *Wellcome Research Laboratories, Research Triangle Park, NC 27709;* and the ‡ *Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, U.K.* Accepted for publication September 21,1981.

Abstract □ A radioimmunoassay for the quantitation of meobentine sulfate, a novel antidysrhythmic and antifibrillatory agent in biological fluids, is described. Antisera were raised in rabbits in response to immunization with a conjugate of bovine serum albumin and a meobentine analog with a propionic acid sidechain *ortho* to the methoxyl group. These antisera have low affinities for *N*- and *O*-desmethylmeobentine metabolites, which show less than 5% cross-reaction in radioimmunoassay procedures employing either tritiated or radioiodinated radioligands. The radioimmunoassay using [^{125}I]meobentine was capable of detecting <0.4 ng/ml (40-pg mass) of meobentine. This assay was used to demonstrate the absorption of meobentine in humans after oral administration and also permitted studies of meobentine sulfate disposition in human plasma following two (2.5 and 5 mg/kg) oral doses. Mean peak meobentine concentrations in plasma occurred 3 hr postdose in both cases and were 230 and 451 ng/ml following the 2.5- and 5-mg/kg doses, respectively. The approximate mean terminal half-life after all treatments was 12 hr.

Keyphrases □ Meobentine sulfate—plasma levels in humans, radioimmunoassay □ Bioavailability—plasma levels of meobentine sulfate in humans determined by radioimmunoassay □ Radioimmunoassay—plasma levels of meobentine sulfate in humans

Meobentine sulfate [bis-(*N*-4-methoxybenzyl-*N′*-*N″*,dimethylguanidinium)sulfate] possesses marked antidysrhythmic properties against arrhythmias induced by ouabain and those induced by coronary artery ligation in dogs (1). It has been demonstrated that meobentine causes a significant incidence of spontaneous recovery from electrically induced fibrillation in the dog[1]. The electrophysiological properties of meobentine have been studied (2). Although very close in structure to the hypotensive agent, bethanidine (3), meobentine is not a neuronal blocking agent and thus, does not decrease blood pressure in animals when administered intravenously at effective antidysrhythmic doses (1). In this respect, meobentine also appears to be superior to the quaternary ammonium compound bretylium tosylate, which is indicated only for treatment of life-threatening ventricular arrhythmias (4) due to its severe hypotensive side effects.

The clinical safety and efficacy of meobentine sulfate when administered to humans by oral and parenteral routes are currently under study. Pharmacokinetic studies of meobentine in animals and humans, which are needed to facilitate the evaluation of meobentine in current clinical trials, require adequately sensitive and specific procedures for the determination of the drug in body fluids. Earlier gas and thin-layer chromatographic techniques lacked sensitivity, while administration and quantitation of radiolabeled meobentine is impractical for extensive pharmacokinetic studies in humans.

[1] W. Wastila *et al.*, submitted to *J. Pharm. Pharmacol.*

0022-3549/ 82/ 0600-0665$01.00/ 0
© 1982, American Pharmaceutical Association