Biochemical Pharmacology, Vol. 32, No. 3, pp. 547–551, 1983.
Printed in Great Britain.

0006-2952/83/030547-05 $03.00/0

INTERACTION OF CAPSAICINOIDS WITH DRUG-METABOLIZING SYSTEMS

RELATIONSHIP TO TOXICITY

MATTHEW S. MILLER,* KLAUS BRENDEL, THOMAS F. BURKS and I. GLENN SIPES
Program in Pharmacology and Toxicology, University of Arizona Health Sciences Center, Tucson, AZ 85724, U.S.A.

(*Received* 7 *January* 1982; *accepted* 23 *July* 1982)

Abstract—The interaction of capsaicin with microsomal drug-metabolizing systems was assessed to determine the role that bioactivation of capsaicin may play in the induction of hepatotoxicity and neurotoxicity. Capsaicin produced a type I spectral change in rat hepatic microsomes in a high affinity (K_s = 8 μM) concentration-dependent manner and was approximately equipotent with SKF-525A in inhibiting ethylmorphine demethylation. Capsaicin (10 mg/kg, s.c.) inhibited biotransformation *in vivo* as measured by prolongation of pentobarbital sleep time. Reactive metabolites of capsaicin were studied using [^{3}H]dihydrocapsaicin. [^{3}H]Dihydrocapsaicin bound irreversibly to hepatic microsomal protein after *in vitro* incubation or *in vivo* administration. No binding was observed in spinal cord or brain. Although the bioactivation and subsequent covalent binding of capsaicin equivalents may initiate events associated with the hepatotoxicity of capsaicin, it appears that capsaicin-induced neuropathy does not involve covalent interactions with neuroproteins in spinal cord or brain.

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), the principle pungent and irritating component of hot peppers, is thought to produce hepatic necrosis following repeated administration [1]. In addition, administration of capsaicin or the capsaicin analog dihydrocapsaicin (8-methyl-*N*-vanillyl-nonanamide) to rats or guinea pigs results in a specific neuropathy characterized by chemogenic analgesia and depletion of the putative peptide neurotransmitter, substance P, from primary afferent neurons in dorsal root ganglia and dorsal spinal cord [2–4]. The mechanism by which capsaicin may produce hepatocellular damage and primary sensory afferent neuron dysfunction is unknown. We have investigated the interactions of capsaicin and dihydrocapsaicin with drug-metabolizing systems in order to assess the role that bioactivation may play in the etiology of capsaicin-induced toxicities.

METHODS

Animals. Adult female Sprague–Dawley rats weighing approximately 200 g were used in all experiments. Animals were housed in metal hanging cages with free access to food and water. Temperature was maintained at 20–23°, and light dark cycles were alternated at 12-hr intervals. All animals were killed by decapitation.

Microsome preparation. Immediately following decapitation, the liver of each animal was perfused through the hepatic portal vein with ice-cold 0.05 M Tris–HCl buffer, pH 7.4, containing 1.15% KCl. Livers were removed, cut into small pieces, and homogenized with a Potter–Elvehjem glass–Teflon homogenizer in 3 vol. of 0.05 M Tris–HCl buffer, pH 7.4, with 1.15% KCl. The homogenate was centrifuged at 15,000 *g* for 20 min at 4°. The supernatant fraction was removed and centrifuged at 100,000 *g* for 50 min. The resulting microsomal pellet was washed by resuspension in 0.05 M Tris–HCl, pH 7.4, with 1.15% KCl followed by recentrifugation at 100,000 *g* for 50 min. The washed microsomal pellet was suspended in Tris–HCl buffer, pH 7.4, with 1.15% KCl. A portion of the microsomes was resuspended in 0.1 M phosphate buffer, pH 7.4, for determination of spectral interactions. All steps were carried out at 4°. Protein concentration was determined by the biuret method [5].

Spectral interactions. Phosphate-buffered microsomal suspensions were diluted to a protein concentration of 2.0 mg/ml. Spectra were measured at room temperature in 1-cm cells with a Beckman Acta CIII dual beam spectrophotometer. Difference spectra were obtained by the addition of microliter quantities of capsaicin solutions, prepared in distilled water, to the sample cuvette. The reference cuvette received an equal volume of distilled water. Difference spectra were recorded between 350 and 500 nm. The extent of spectral change was considered to be the absorption difference between wavelengths of minimum and maximum absorption. The K_s for spectral interaction was calculated by Eadie–Hofstee analysis.

Microsomal demethylase assay. Capsaicin (1 mM) and/or ethylmorphine (1 mM) were incubated for 15, 30 and 60 min at 37° with microsomal protein (2 mg) and NADPH-generating system consisting of NADP (0.8 mg), glucose-6-phosphate (3 mg) and glucose-6-phosphate dehydrogenase (10 units) in a

* Address correspondence to: Matthew S. Miller, Ph.D., Institute of Neurotoxicology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461, U.S.A.

final volume of 2.0 ml. The NADPH-generating system was omitted from control incubations. All determinations were conducted in duplicate. Formaldehyde, produced as a result of demethylation, was measured by the method of Nash [6], as modified by Cochin and Axelrod [7].

Inhibition studies. Ethylmorphine (0.25, 0.5, 1.0 and 2.0 mM) was incubated at 37° with microsomal protein (2 mg) and capsaicin (0, 25 and 50 μM) or SKF-525A (25 μM) in a total volume of 2.0 ml. After 15 min, formaldehyde production was assessed by the method of Nash [6] as modified by Cochin and Axelrod [7] and data were analyzed by Eadie–Hofstee analysis.

Pentobarbital sleep time. Pentobarbital sleeping time was measured in female Sprague–Dawley rats (250 g). Sodium pentobarbital (30 mg/kg, i.p.) was administered to animals 6 hr after treatment with capsaicin (10 mg/kg, s.c.) or capsaicin vehicle (10% ethanol, 10% Tween 80, 80% saline; 1.0 ml/kg, s.c.). Time to regain the righting reflex was measured. Upon awakening, animals were killed, and serum pentobarbital levels were determined by the method of Cooper and Brodie [8]. Data were analyzed by Student's *t*-test.

Synthesis of dihydrocapsaicin. Capsaicin was suspended in methanol within a closed hydrogen-filled flask attached to a hydrogen-filled manometer. One milliliter of 5% palladium on charcoal, in methanol (200 mg/ml) previously equilibrated with hydrogen, was added to the flask by syringe through a Teflon septum. The suspension was stirred constantly, and uptake of hydrogen was measured. The reaction was considered terminated when hydrogen uptake ceased. The suspension was then centrifuged at 5000 *g* for 20 min to remove catalyst, and dihydrocapsaicin was recrystallized from *n*-hexane. Contamination of dihydrocapsaicin with capsaicin was estimated to be less than 5% by melting point analysis and chemical ionization mass-spectroscopy.

Radiolabeled dihydrocapsaicin was similarly prepared by the addition of carrier-free tritium gas to the reaction flask through a specially prepared glass side arm. Specific activity of [^{3}H]dihydrocapsaicin was 674 mCi/mmole.

Binding to microsomal protein. Dihydrocapsaicin (1 mM) containing 1 μCi of [^{3}H]dihydrocapsaicin was incubated with NADPH-generating system and microsomal protein (2 mg) for 15, 30, 60 and 90 min at 37° in a total volume of 2.0 ml. Nonenzymatic background binding was determined by use of microsomes that had been boiled for 5 min prior to incubation or by omission of the NADPH-generating system from the incubations. These treatments gave essentially the same level for background binding. Incubations were terminated by the addition of 2 ml of ice-cold ethanol. Unbound radioactivity was then extracted with the following series of solvents; chloroform/ethanol (1:3), 100% ethanol, 0.01 M HCl, methanol/ether (3:1) and acetone. This solvent series extracted 100% of [^{3}H]dihydrocapsaicin that had been added to liver, brain and spinal cord homogenates. The resulting protein pellet was digested with 1 N sodium hydroxide and neutralized with 1 N perchloric acid, and tritium content was determined by liquid scintillation counting. Protein content of the digestate was determined by the biuret method [5].

Capsaicin and dihydrocapsaicin metabolites. Capsaicin (1 mM) and dihydrocapsaicin (1 mM) containing 1 μCi of [^{3}H]dihydrocapsaicin were incubated with microsomal protein (2 mg) with and without NADPH-generating system at 37° for 15 min in a total volume of 2 ml. Upon completion, incubations were frozen immediately in a dry-ice methanol bath and lyophilized to dryness. The lyophilizate was extracted with 500 μl methanol, and an aliquot of the extract was applied to LK5D (80 Å) silica gel thin-layer chromatography plates. Plates were eluted with ethylacetate–acetic acid (9:1) and dried. Phenols were visualized by spraying plates with 1% (w/v) dichloroquinone-4-chloroimide (Gibb's reagent) in methanol followed by exposure to ammonia vapor for 60 sec. Plates containing radioactivity were subsequently scored in 1-cm increments and scraped for liquid scintillation counting.

In vivo *dihydrocapsaicin binding.* Female Sprague–Dawley rats (200 *g*) received a single dose of dihydrocapsaicin (10 mg/kg, s.c.) containing 5 μCi [^{3}H]dihydrocapsaicin. Liver, brain and spinal cord were removed 3, 6, 12 and 24 hr after treatment. Tissues were homogenized in 0.05 Tris–HCl buffer, pH 7.4, containing 1.15% KCl. An aliquot of each homogenate was assayed for total radioactivity by scintillation counting following digestion with 1 N sodium hydroxide and neutralization with 1 N perchloric acid. Bound radioactivity was then measured in an aliquot of each homogenate as previously described. The remainder of each homogenate was centrifuged at 10,000 *g* for 20 min. The supernatant fraction (S-9) containing cytosolic and microsomal protein was assayed for bound radioactivity by the solvent extraction procedure described previously.

RESULTS

Capsaicin interacted with rat hepatic microsomes to produce a type I difference spectrum. The extent of the interaction was concentration-dependent. Eadie–Hofstee analysis revealed a K_s for spectral interaction of capsaicin with hepatic microsomes of approximately 8 μM (Fig. 1).

Although rat hepatic microsomes readily demethylated ethylmorphine, no demethylation of capsaicin was detectable (Fig. 2). Addition of equimolar quantities of capsaicin to ethylmorphine incubations resulted in complete inhibition of ethylmorphine demethylation (Fig. 2). More detailed inhibition studies revealed that capsaicin inhibited ethylmorphine demethylation in a manner which displayed both competitive and noncompetitive characteristics (Table 1). The potency with which capsaicin inhibited ethylmorphine demethylation was approximately equal to that of SKF-525A (Table 1).

Inhibition of biotransformation *in vivo* by capsaicin was investigated by determining sleeping time in response to sodium pentobarbital (30 mg/kg, i.p.). A single treatment with capsaicin (10 mg/kg, s.c.) significantly prolonged pentobarbital-induced sleeping time (284 ± 17 min, capsaicin-treated vs 133 ± 9 min, control; $P < 0.001$). No differences in serum pentobarbital levels were detected ($P > 0.2$).

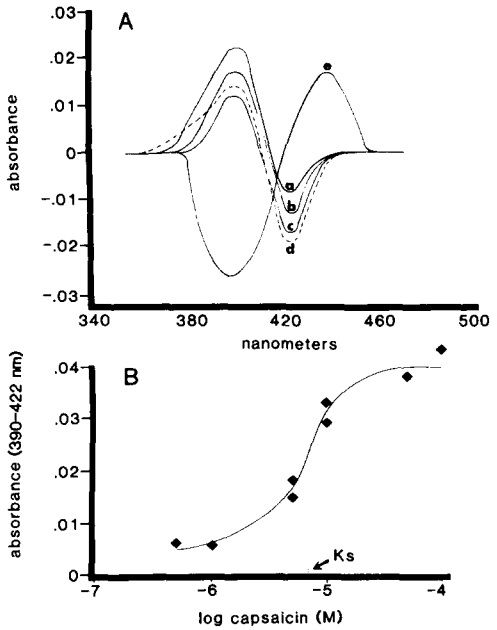

Fig. 1. Spectral interaction of capsaicin with rat hepatic microsomes. (A) Type I difference spectra which resulted when capsaicin [5×10^{-6} M (a), 5×10^{-5} M (b), and 5×10^{-4} M (c)] interacted with hepatic microsomes. Aminopyrine (7 mM) (d) and aniline (7 mM) (e) were included as controls. (B) Concentration-dependent nature of the spectral interaction. The K_s (concentration for one-half maximum absorbance) equalled 8 μM.

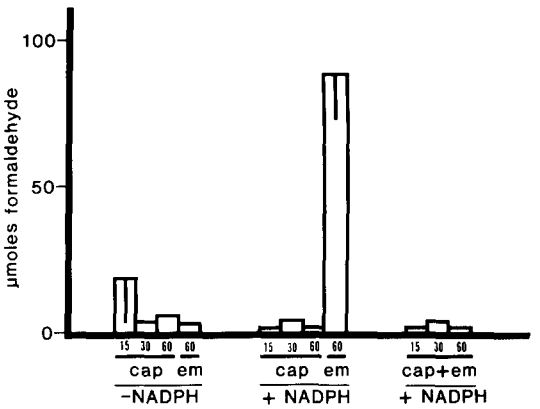

Fig. 2. Formaldehyde production by rat liver microsomes incubated with 1 mM capsaicin (cap) and/or 1 mM ethylmorphine (em) for 15, 30 and 60 min with and without the NADPH-generating system. No detectable demethylation of capsaicin occurred, and the addition of capsaicin completely inhibited demethylation of ethylmorphine. Values are means ± S.E. N = three incubations per time point.

Since radiolabeled capsaicin is not available, covalent binding of capsaicinoids was investigated using a radiolabeled active analog of capsaicin, [^{3}H]dihydrocapsaicin [2, 3], as substrate for microsomal biotransformation systems. [^{3}H]Dihydrocapsaicin interacted in a non-extractable manner with microsomal protein at a rate of approximately 60 pmoles per mg protein per hr (Fig. 3). Heat denaturation, removal of NADPH-generating system, addition of reduced glutathione (1 mM), or addition of equimolar SKF-525A (1 mM) significantly inhibited the apparent covalent binding of [^{3}H]dihydrocapsaicin to microsomal protein (Fig. 3).

Analysis of microsomal metabolites of capsaicin and dihydrocapsaicin by thin-layer chromatography revealed a single metabolite peak of radioactivity for

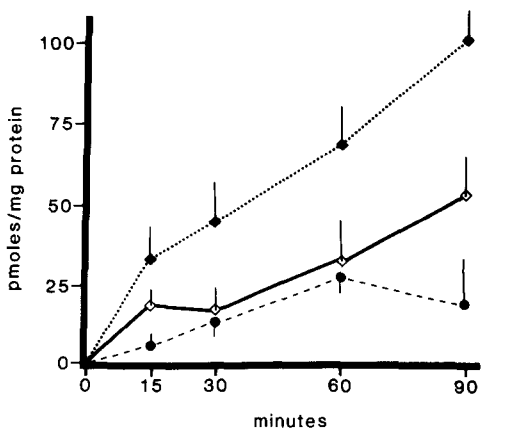

Fig. 3. Covalent binding of [^{3}H]dihydrocapsaicin to microsomal protein during *in vitro* incubation. [^{3}H]Dihydrocapsaicin bound to hepatic microsomes in an unextractable manner at a rate of approximately 60 pmoles per hr per mg protein (. . . . .). Binding was inhibited by omission of the NADPH-generating system or by prior heat denaturation of microsomal protein. Addition of reduced glutathione (1 mM) (– – – –) or SKF-525A (1 mM) (———) markedly inhibited binding. Points are means ± S.E. after subtraction of value for heat-denatured microsomes. N = three incubations per time point.

Table 1. Effects of capsaicin and SKF-525A on Eadie–Hofstee kinetic constants for the production of formaldehyde resulting from demethylation of ethylmorphine by rat liver microsomes*

Inhibitor concentration (μM)				
Capsaicin	SKF-525A	r	K_m (mM)	V_{max} (nmoles/mg protein)
		0.999	1.83 ± 0.05	6.0 ± 0.2
25		0.999	2.78 ± 0.03†	5.3 ± 0.4†
50		0.987	3.22 ± 0.04†	4.6 ± 0.2†
	25	0.999	2.85 ± 0.07†	5.4 ± 0.3†

* Values are means of four experiments ± S.E.
† $P < 0.05$ vs no inhibitor by analysis of variance and Scheffe's test.

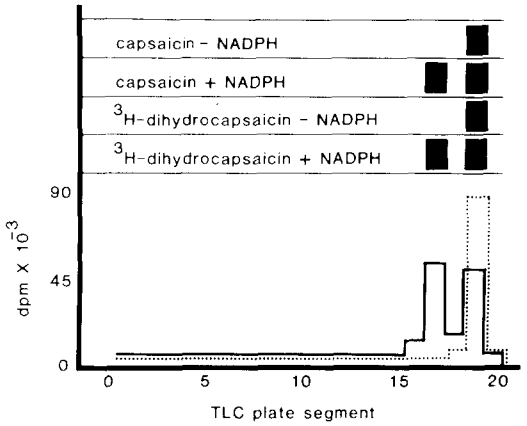

Fig. 4. Thin-layer chromatography of capsaicin, [^{3}H]dihydrocapsaicin and metabolites. Microsomal incubations of capsaicin (1 mM) or [^{3}H]dihydrocapsaicin (1 mM) with and without NADPH-generating system were chromatographed on LK5D (80 Å) silica gel plates. Mobile phase was ethylacetate–acetic acid (9:1). Upper frames show spots detected after plates were treated with 1% 2,6-dichloroquinone-4-chloroimide and ammonia. Lower frame shows radioactivity profile following chromatography of microsomal incubations of [^{3}H]dihydrocapsaicin with (———) or without (– – – –) NADPH-generating system.

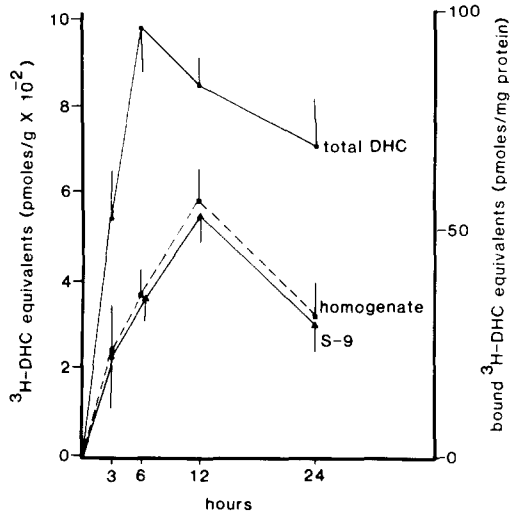

Fig. 6. Covalent binding of [^{3}H]dihydrocapsaicin to rat liver protein. Animals received [^{3}H]dihydrocapsaicin (10 mg/kg, s.c.; 5 μCi). Non-extractable radioactivity was determined in protein from whole liver homogenates (■, – – –) and S-9 fractions (▲, ——) at various times after dihydrocapsaicin treatment. Levels of total hepatic radioactivity are also shown (●, ——). All values are means ± S.E. N = five animals per group.

each substrate (Fig. 4). The presence of these peaks, which comigrated, was dependent upon the presence of an active microsomal metabolizing system.

The distribution to selected tissues and *in vivo* covalent binding of [^{3}H]dihydrocapsaicin was investigated in rats following administration of dihydrocapsaicin (10 mg/kg, s.c.) containing 5 μCi of [^{3}H]dihydrocapsaicin. The maximum concentration of [^{3}H]dihydrocapsaicin equivalents was found in the liver 6 hr after treatment. Brain and spinal cord concentrations did not approach maximum values until 24 hr after treatment (Fig. 5). [^{3}H]Dihydrocapsaicin bound covalently (i.e. non-extractable) to hepatic protein, attaining a maximum value of approximately 55 pmoles/mg protein 12 hr after treatment. Binding to total hepatic protein was equal to that measured in the S-9 fraction (Fig. 6). No non-extractable radioactivity was detected in brain or spinal cord homogenates.

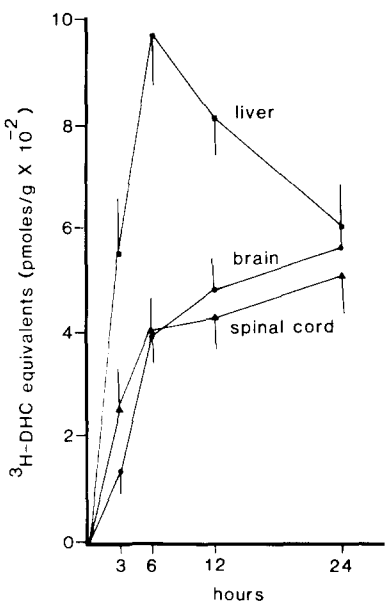

Fig. 5. Distribution of radioactivity to brain, spinal cord and liver of rats treated with [^{3}H]dihydrocapsaicin 10 mg/kg, s.c.; 5 μCi).

DISCUSSION

Capsaicin, the principle pungent constituent of hot peppers, has been shown in this study to interact with hepatic drug-metabolizing systems in a high affinity, concentration-dependent manner. This high affinity interaction suggests that capsaicin, and its analogs, may be potent inhibitors of biotransformation systems. This postulate is supported by the profound inhibition of ethylmorphine demethylation induced by capsaicin in *in vitro* microsomal incubations as well as by the prolongation of sodium pentobarbital sleeping time produced by administration of capsaicin to rats *in vivo*. Since capsaicin inhibited ethylmorphine demethylation *in vitro* in a manner which was not purely competitive, it was not possible to precisely calculate a K_I value. However, capsaicin at a concentration of 25 μM inhibited ethylmorphine demethylation to approximately the same extent as did equimolar SKF-525A. This suggests that capsaicin is as potent an inhibitor of microsomal cytochrome P-450 mediated biotransformation reactions as is SKF-525A. The pharmacological significance of the potent inhibition of biotransformation

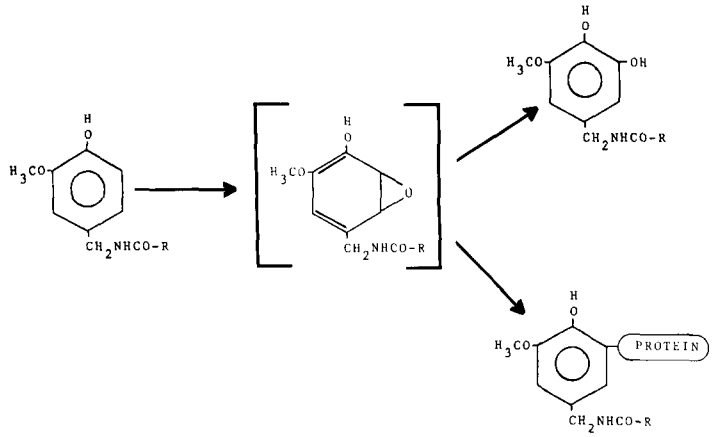

Fig. 7. Postulated route of capsaicinoid biotransformation by rat hepatic microsomes. R denotes the $(CH_2)_4CH{=}CHCH(CH_3)_2$ group of capsaicin or the $(CH_2)_6CH(CH_3)_2$ group of dihydrocapsaicin.

produced by capsaicin is currently unknown. However, the potential for altered biotransformation of therapeutic agents as a result of dietary capsaicin should be considered.

Data obtained with [^{3}H]dihydrocapsaicin suggest that capsaicinoids are bioactivated to a species which is capable of covalently interacting with cellular macromolecules. This may explain, at least in part, the partially noncompetitive manner in which capsaicin inhibits ethylmorphine demethylation. Thin-layer chromatographic data obtained for the metabolites of capsaicin and [^{3}H]dihydrocapsaicin are consistent with the finding of Lee and Kumar [9] who demonstrated that capsaicinoids are biotransformed *in vitro* solely by ring hydroxylation to produce *N*-(4,5-dihydroxyl-3-methoxybenzyl)-acylamides. The apparent covalent interaction of dihydrocapsaicin with microsomal protein suggests that hydroxylation of capsaicinoids proceeds through a reactive intermediate, most likely an epoxide (Fig. 7). The inhibition of [^{3}H]dihydrocapsaicin binding to microsomal protein observed in the presence of reduced glutathione further supports the existence of a reactive metabolite of capsaicinoids. The significance of covalent binding of capsaicinoids to hepatic protein has yet to be established. However, detection of radioactivity bound covalently (i.e. non-extractable) to hepatic protein following administration of [^{3}H]dihydrocapsaicin *in vivo* suggests that hepatic damage reported after long-term hot pepper consumption [1] may result from covalent interactions between capsaicinoids and hepatic macromolecules.

Parenteral administration of capsaicinoids has been shown to produce depletion of the putative peptide neurotransmitter substance P [10, 11] from primary afferent neurons which terminate in the substantia gelatinosa of the spinal cord [3]. Substance P containing neurons in the hypothalamus and corpus striatum are unaffected by capsaicinoid treatment [3]. Data obtained with [^{3}H]dihydrocapsaicin have demonstrated that radioactivity readily gains access to the brain, presumably including the hypothalamus and corpus striatum, after parenteral administration. Therefore, the apparent resistance of hypothalamic and striatal substance P containing neurons to the action of capsaicinoids appears not to be a result of differential distribution of capsaicinoids. Finally, the absence of detectable covalent binding of [^{3}H]dihydrocapsaicin in brain or spinal cord strongly suggests that the mechanism by which capsaicinoids deplete the neuropeptide substance P from the spinal cord does not involve the bioactivation and covalent binding of capsaicinoids to neuronal proteins.

Acknowledgements—This work was supported in part by USPHS Grants ES 82130, DA 02173, NS 15420 and a University of Arizona Graduate Student Development Award to M.S.M.

REFERENCES

1. S. O. Lee, *Korean J. intern. Med.* **6**, 383 (1963).
2. M. S. Miller, S. H. Buck, K. Brendel and T. F. Burks, *Pharmacologist* **22**, 206 (1980).
3. M. S. Miller, S. H. Buck, R. Schnellmann and T. F. Burks, *Fedn Proc.* **40**, 274 (1980).
4. T. F. Burks, S. H. Buck, M. S. Miller, P. P. Deshmukh and H. I. Yamamura, *Proc. west. Pharmac. Soc.* **24**, 353 (1981).
5. A. G. Gornall, C. S. Bardawill and M. D. David, *J. biol. Chem.* **177**, 751 (1949).
6. T. Nash, *Biochem. J.* **55**, 16 (1953).
7. J. Cochin and J. Axelrod, *J. Pharmac. exp. Ther.* **125**, 105 (1959).
8. J. R. Cooper and B. B. Brodie, *J. Pharmac. exp. Ther.* **114**, 409 (1955).
9. S. S. Lee and S. Kumar, in *Microsomes, Drug Oxidations, and Chemical Carcinogenesis* (Eds. M. J. Coon, A. H. Conney, R. W. Estabrook, H. V. Gelboin, J. R. Gillette and P. J. O'Brien), Vol. 2, pp. 1009–12. Academic Press, New York (1980).
10. G. Jansco and E. Kinihar, *Neurobiology* **5**, 42 (1975).
11. J. L. Henry, *Brain Res.* **114**, 439 (1976).