Title	ABSORPTION OF PUNGENT PRINCIPLE OF HOT PEPPER AND ITS EFFECTS ON LIPID METABOLISM IN RATS(Dissertation_)
Author(s)	Kawada, Teruo
Citation	Kyoto University ( )
Issue Date	1984-03-23
URL	http://dx.doi.org/10.14989/doctor.k3128
Right	
Type	Thesis or Dissertation
Textversion	author

ABSORPTION OF PUNGENT PRINCIPLE

OF HOT PEPPER AND ITS EFFECTS

ON LIPID METABOLISM IN RATS

TERUO KAWADA

1984

CONTENTS

ABBREVIATIONS

CAP, capsaicin [N-(4-hydroxy-3-methoxybenzyl)-8-methylnon-<u>trans</u>-
6-enamide]

DC, dihydrocapsaicin [N-(4-hydroxy-3-methoxybenzyl)-8-methylnon-
amide]

Iso-$C_{10:0}$, 8-methyl nonanoic acid

n-$C_{11:0}$, undecanoic acid

TLC, thin-layer chromatography

HPTLC, high-performance thin-layer chromatography

HPLC, high-performance liquid chromatography

GC, gas chromatography

GC-MS, gas chromatography-mass spectrometry

SDS, sodium dodecylsulfate

G-6-PDH, glucose-6-phosphate dehydrogenase

HADH, beta-hydroxyacyl-CoA dehydrogenase

HSL, hormone sensitive lipase

LPL, lipoprotein lipase

i.p., intraperitoneally

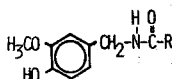s.c., subcutaneously

p.o., per os

i.v., intravenous

i.m., intramuscular

INTRODUCTION

A spice is "that which enriches or alters the quality of a
thing, especially in a small degree, as spice alters the taste of
food; that which gives zest or pungency; a piquant or pleasing
flavoring; as variety is the spice of life"(1).

Hot pepper is one of the popular spices and used for various
cooking in the world. For example, Thai people have been taken the
pungent principle of hot pepper about 50-60 mg per day for each
person(2). In our country, there has been an increasing
consumption of hot pepper with the variation of our food life.

The pungent principle of hot pepper gives a general name to
capsaicinoids, the structure of which are acid amides of C_9-
C_{11} branched-chain fatty acids and vanillylamine(3)(Fig.).Five
analogues occuring in nature have been reported(3-6): capsaicin
[N-(4-hydroxy-3-methoxybenzyl)-8-methylnon-trans-6-enamide)],
dihydrocapsaicin [N-(4-hydroxy-3-methoxybenzyl)-8-methylnonamide],
nordihydrocapsaicin [N-(4-hydroxybenzyl)-7-methyloctamide], homo-
dihydrocapsaicin [N-(4-hydroxy-3-methoxybenzyl)-9-methyldecamide],
and homocapsaicin [N-(4-hydroxy-3-methoxybenzyl)-9-methyldec-
trans-7-enamide]. Recently, novel but minor analogues having
ante-iso branched-chain fatty acyl moieties or shorther straight-
chain fatty acyl moieties have also been reported(7). Although
there have been many reports on the methodology of the quantita-

H_3CO —[ring]— CH_2-N-C-R with H and O, HO on ring

R	acyl moiety	name
CH_3-CHCH=CH$(CH_2)_4$- (with t) and CH_3	iso-$C_{10:1}$	Capsaicin(CAP)
CH_3-CH$(CH_2)_6$- and CH_3	iso-$C_{10:0}$	Dihydrocapsaicin(DC)
CH_3-CH$(CH_2)_5$- and CH_3	iso-$C_{9:0}$	Nordihydrocapsaicin(NDC)
CH_3-CHCH=CH$(CH_2)_5$- (with t) and CH_3	iso-$C_{11:1}$	Homocapsaicin(HC)
CH_3-CH$(CH_2)_7$- and CH_3	iso-$C_{11:0}$	Homodihydrocapsaicin(HDC)

Fig.1. STRUCTURE OF PUNGENT PRINCIPLE OF HOT PEPPER

tive analysis of capsaicinoids(8–13) and the plant biochemistry (3,5,14–20), very few have been reported from the food scientific point of view(21–25). Thus detailed knowledge on the absorption and metabolism of pungent principle of hot pepper, and its effects on the lipid metabolism in the animal has been limited.

In this thesis, in order to elucidate effects of the pungent principle in the animal, the author first describes the quantitative microanalysis of the pungent principle by HPTLC and HPLC. Secondly, the gastrointestinal absorption of the pungent principle in vivo and in situ rats is described. Thirdly, the metabolism of the pungent principle in vivo and in vitro rats is described. Fourthly, the effects of the pungent principle on lipid metabolism in high fat-fed rats are described. Finally, in vivo analyses for the pungent principle to enhance the lipid metabolism of rats by the measurment of the energy metabolism are described, then a possible effect mechanism of the pungent principle on the lipid

metabolism of rats is also proposed.

REFERENCES

1. F.Rosegarten,Jr. In The Book of Spices, Pyramid

    Communications, INC. New York, 1973, pp 5.

2. Interdepartment Committee on Nutrition for National

    Defense, Nutrition Survey-The Kingdom of Thailand. U.S.

    Government Printing Office, Washington,D.C., 1962, pp 57.

3. D.J.Bennet and G.W.Kirby, J. Chem. Soc., (C), 442(1968).

4. L.Crombie, S.H.Dandegoanker and K.B.Simpson, J. Chem. Soc.

    1025(1955).

5. E.Leete and M.C.Louden, J. Amer. Chem. Soc., 90, 6837

    (1968).

6. S.Kosuge, Y.Inagaki and K.Uehara, Nippon Nogeikagaku

    Kaishi, 32, 578(1958).

7. J.Jurentisch, M.David, F.Hersch and W.Kubelka, Planta Med.

    36, 61(1979).

8. J.I.Suzuki, F.Tausing and R.E.Morse, Food Technol., 11,

    100(1957).

9. J.J.Dicecco, J. Ass. Offic. Anal. Chem., 62, 988(1979).

10. P.H.Todd,Jr., M.G.Bensinger and T.Biftu, J.Food Sci., 42

    660(1977).

11. Y.Masada, K.Hashimoto, T.Inoue and M.Suzuki, J. Food Sci.

    36, 858(1971).

12. K.Iwai, T.Suzuki, H.Fujiwake and S.Oka, J. Chromatogr., 172, 303(1979).

13. F.Hersch and J.Jurentisch, Chromatographia, 12, 647 (1979).

14. W.R.Bowman, I.T.Brucfe and G.W.Kirby, J. Chem. Soc., (D) 1075(1969).

15. K.Iwai, K.R.Lee, M.Kobashi and T.Suzuki, Agric. Biol. Chem., 41, 1873(1977).

16. K.Iwai, K.R.Lee, M.Kobashi, T.Suzuki and S.Oka. ibid., 42, 201(1978).

17. H.Fujiwake, T.Suzuki and K.Iwai, ibid., 44, 2097(1980).

18.   idem., Plant & Cell Physiol., 21, 1023(1980).

19. T.Suzuki, H.Fujiwake and K.Iwai, ibid., 21, 839(1980).

20. H.Fujiwake, T.Suzuki and K.Iwai Agric. Biol. Chem., 46, 2685(1982).

21. S.O.Lee, Korean J. Intern. Med., 6, 383(1963).

22. W.Nopanitaya and Y.Nye, Toxicol. Appl. Pharmacol., 30, 149(1974).

23. P.Ki, J.A.Negulesco and M.Murnama, IRCS Med. Sci., 10, 446(1982).

24. K.Sambaiah, M.N.Satyanarayana and M.U.L.Rao, Nutr. Rep. Int 18, 521(1978).

25. K.Sambaiah and M.N.Satyanarayana, J. Food Sci. Technol., 19, 30(1982).

CHAPTER 1

QUANTITATIVE MICROANALYSIS OF PUNGENT PRINCIPLE OF
HOT PEPPER BY HPTLC AND HPLC[i]

With respect to the analyses of pungent principle of hot pepper, many methods using spectrometry[1,2], paper chromatography[3], thin-layer chromatography[4,5], gas-chromatography[6–8], and gas chromatography-mass spectrometry[9,10] have been reported. However, the traditional methods appear to have some disadvantages relating to the reliability of the data, separation ability, running time and cost. On the other hand, high-performance thin-layer chromatography(HPTLC) and high-performance liquid chromatography(HPLC) offer a new approach which greatly simplifies sample treatment and saves time.

In the course of my studies of the absorption and metabolism of the pungent principle of hot pepper, it became necessary to determine the individual compounds in commercial "Capsaicin" separately. The present chapter deals with simple and rapid microanalysis of the pungent principle by HPTLC and HPLC.

EXPERIMENTAL

Materials

Reversed-phase thin-layer plates(RP-8) were purchased from E. Merck(Darmstadt, G.F.R.). Authentic capsaicin was purchased from

Sigma(St.Louis,MO, U.S.A.). cis-Capsaicin, dihydrocapsaicin, nordihydrocapsaicin, cis-homocapsaicin and homodihydrocapsaicin were synthesized according to the method as described by Rangoon-wala and Seitz(**11**). Naturally occuring the pungent principle was obtained from red chilli fruit(Capsicum annuum var. annuum cv. Red Chilli) by extraction with ethyl acetate(12). Other chemicals were guaranteed reagent-grade.

One-dimensional HPTLC

A 5-200 nl volume of an 0.01% ethyl acetate solution of indi-vidual pungent principle or a mixture were applied to the RP-8 plate(10x10 cm) with the aid of a Nanoapplicator(Atto Instruments, Tokyo). After the samples had been spotted, the plate was devel-oped in a filter-paper lined chamber with 0.05 M each of silver nitrate and boric acid in 85% methanol for 5 cm without pre-conditioning. After development, the plate was dried at room temperature. Detection was accomplished by locating the blue spots after spraying with 0.1% 2,6-dichloroquinone 4-chloroimide in methanol and subsequent exposure to ammonia vapour.

For the calibration of capsaicin and its analogues, the absorption of the located spots was measured with a Shimadzu Type CS-910 dual-wavelength thin-layer chromatoscanner, (Shimadzu Seisaku-sho,Kyoto). The absorption of the pungent principle was measured at 610 nm for the sample side and 710 nm for the refer-ence side by the "zig-zag" mode scanning. The slit width and

height were 1.25 mm. Measurement was carried out within 30 min after the location of the spots.

Two-dimensional HPTLC

The RP-8 plate loaded with the mixture of pungent principle was developed first with 85% methanol, then air-dried prior to the second development with 0.05 M each of silver nitrate and boric acid in 85% methanol. The solvents were developed 7 cm in both directions. Detection was accomplished with the same reagents as used in one-dimensional HPTLC. The separations were repeated at least three times in each experiment.

HPLC

The system for HPLC consisted of a Shimadzu Model LC-3A, 20x 4.2 mm I.D. packed with 30 µm of LiChrosorb RP-2(E.Merck) as a guard column, Cosmosil 5C$_8$ (Nakarai Chemicals, Kyoto) of 150 mm x4.6 mm I.D. for the separation column, Shimadzu Model SPD-2A uv detector (280 nm), and E1A microcomputer. The mobile phase used for separating capsaicin (CAP) and dihydrocapsaicin (DC) was 75% methanol. The flow rate was kept at 1.0 ml/min.

RESULTS AND DISCUSSION

HPTLC

Rf values of capsaicin and its analogues are presented in Table I. When the chromatograms were developed with methanol only, the separation between trans-capsaicin and nordihydrocap-

TABLE I

R_F VALUES OF CAPSAICIN AND ITS ANALOGUES ON RP-8 HPTLC PLATES DEVELOPED WITH VARIOUS SOLVENT SYSTEMS

Solvent system	cis-CAP*	trans-CAP*	cis-HC*	NDC*	DC*	HDC*	Development time (min)
0.05 M AgNO$_3$ in 90% MeOH	0.59	0.52	0.53	0.47	0.43	0.37	120
0.05 M AgNO$_3$ in 80% MeOH	0.42	–	0.36	0.28	0.23	0.17	150
0.05 M AgNO$_3$ + 0.05 M H$_3$BO$_3$ in 80% MeOH	0.51	0.44	–	0.38	0.32	0.26	90
0.05 M AgNO$_3$ + 0.05 M H$_3$BO$_3$ in 85% MeOH	0.50	0.45	0.45	0.37	0.29	0.24	90
85% MeOH	0.36	0.36	0.29	0.37	0.29	0.23	40
90% MeOH	0.67	0.67	0.62	0.67	0.62	0.57	40

* cis-CAP = cis-capsaicin (synthetic); trans-CAP = trans-capsaicin (synthetic); cis-HC = cis-homocapsaicin (synhtetic); NDC = nordihydrocapsaicin (synthetic); DC = dihydrocapsaicin (synthetic); HDC = homodihydrocapsaicin (synthetic). Each R_F value is the average value obtained from eight analyses and the error for each R_F value was less than 1.5%.
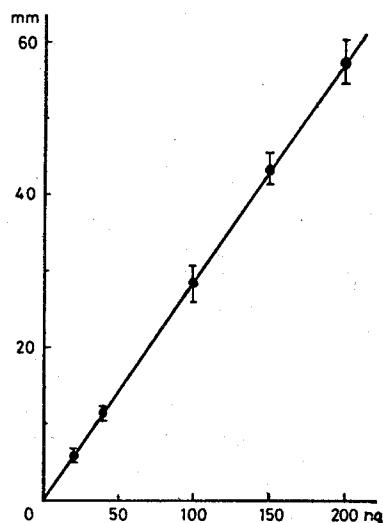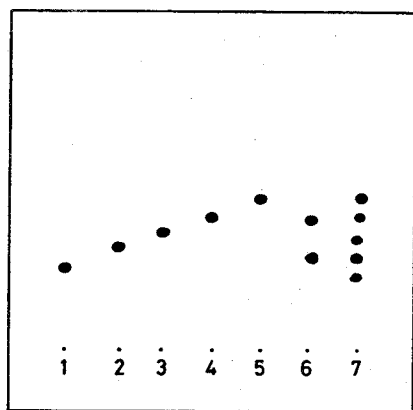
Fig. 1. Reversed-phase HPTLC of naturally occurring and synthetic capsaicin and its analogues on RP-8 plate developed with 0.05 M AgNO$_3$ and 0.05 M H$_3$BO$_3$ in 85% methanol. Spots were located by spraying with 0.1% 2,6-dichloroquinone 4-chloroimide and subsequent exposure to ammonia vapour. 1 = Homodihydrocapsaicin; 2 = synthetic dihydrocapsaicin; 3 = synthetic nordihydrocapsaicin; 4 = synthetic *cis*-homocapsaicin; 5 = synthetic *cis*-capsaicin; 6 = Sigma capsaicin, which was a mixture of capsaicin and dihydrocapsaicin; 7 = mixture of the samples 1–5.

Fig. 2. Calibration graph for capsaicin and its analogues, prepared by measuring the integration heights at 610 nm on a Shimadzu CS-910 dual-wavelength chromatoscanner. The integration heights are average values obtained from seven analyses. Linearity is obtained up to 200 ng ($y = 0.284x + 0.872$, where y is the integration height, and x ng the amount of capsaicin analogue). The calibration graph for *cis*-capsaicin and nordihydrocapsaicin is presented here, but other samples gave essentially the same results.

saicin was unacceptable. However, good separations of capsaicin and its analogues could be obtained by the addition silver nitrate and boric acid(Fig.1).

trans-Capsaicin and cis-homocapsaicin had the same Rf values, but this is not important because the latter dose not occur in nature. All naturally occurring capsaicins and homocapsaicins have a trans configuration. The Rf value of trans-homocapsaicin is not presented here. However, as the Rf value of the synthesized cis-capsaicin was higher than that of trans-
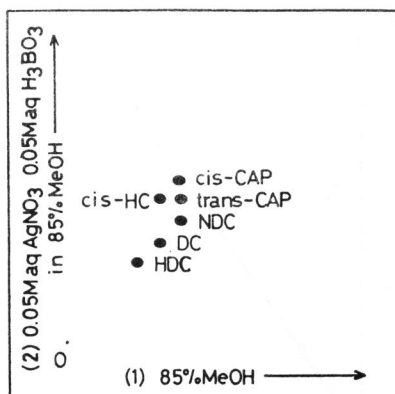
Fig. 3. Two-dimensional RP HPTLC of capsaicin and its analogues. The sample mixture consisting of *trans*-capsaicin (*trans*-CAP), *cis*-capsaicin (*cis*-CAP), dihydrocapsaicin (DC), nordihydrocapsaicin (NDC), homodihydrocapsaicin (HDC) and *cis*-homocapsaicin (*cis*-HC) was applied at the starting point (0). The success of the separation with 85% methanol in the first direction, and 0.05 M each of $AgNO_3$ and H_3BO_3 in 85% methanol in the second can be seen from the chromatogram.
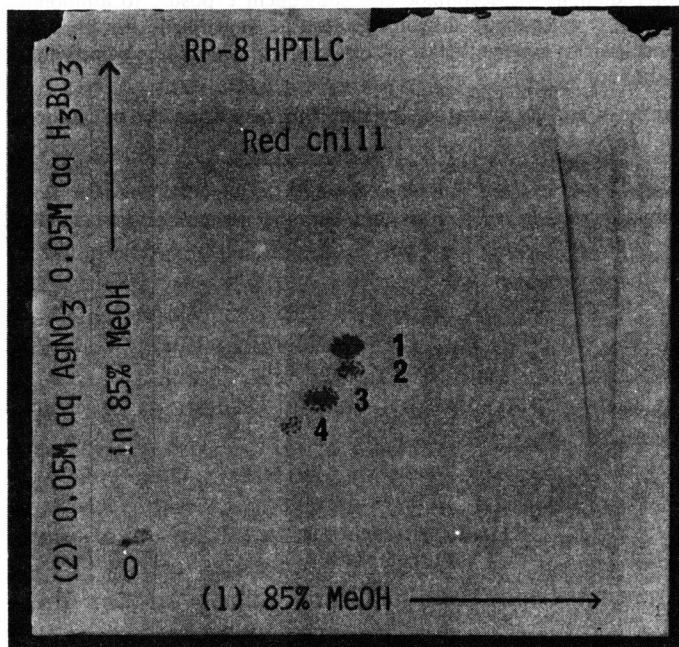

Fig. 4. Two-dimensional RP HPTLC of capsaicin and its analogues extracted from the fruits of red chilli. About 500 ng of capsaicinoids were applied at the starting point (0). 1 = *trans*-Capsaicin; 2 = nordihydrocapsaicin; 3 = dihydrocapsaicin; 4 = homodihydrocapsaicin.

capsaicin, it would be located below cis-homocapsaicin. Even if the Rf value of trans-homocapsaicin were the same as that of cis-homocapsaicin, it would not matter in practice because trans-homocapsaicin has been detected in only trace amounts in most Capsicum species. Linearity was found at least from 20 to 200 ng(Fig.2).

If complete separation is necessary, it is accomplished by two-dimensional reversed-phase (RP) HPTLC. Fig.3 shows a thin-layer chromatogram of naturally occuring and synthetic capsaicin and its analogues accomplished by two-dimensional RP HPTLC. Complete separation between each analogue was achieved.

Fig.4 shows a chromatogram of pungent principle of hot pepper extracted from red chilli. About 500 ng of the pungent principle were applied on the RP-8 plate. Dominant spots of trans-capsaicin and dihydrocapsaicin were observed, together with small spots of nordihydrocapsaicin and homodihydrocapsaicin. Two-dimensional RP HPTLC can be accomplished in 3 hr. One-dimensional RP HPTLC appears to be adequate for routine analysis.

The analysis of capsaicin and its analogues using RP HPTLC is sensitive, and much more convenient than former methods. Application of one-dimensional RP HPTLC to the determination of the content and composition of the pungent principle in some Capsicum species is shown in Table II.

TABLE II

APPLICATION OF HPTLC ON RP-8 PLATES TO THE DETERMINATION OF CONTENT AND COMPOSITION OF CAPSAICINOID IN SOME HOT PEPPER SPECIES PURCHASED FROM DIFFERENT AREAS

ND = not detected; tr = trace (<0.01 mg/dry fruit). The analysis was performed by one-dimensional HPTLC. The non-existence of *trans*-homocapsaicin (*trans*-HC) was confirmed by mass chromatography[12].

Hot pepper	Harvested area	Capsaicinoid [mg/dry fruit] (%)				
		NDC	DC	trans-CAP	HDC	trans-HC
C. annuum var. annuum*	Guizhou (China)	0.18 (8.6)	0.59 (28.2)	1.23 (58.9)	0.09 (4.3)	ND (ND)
C. annuum var. annuum*	Hainan (China)	0.03 (5.9)	0.15 (29.4)	0.32 (62.7)	0.01 (2.0)	ND (ND)
C. annuum var. annuum*	Guangxi (China)	0.04 (5.1)	0.22 (27.8)	0.53 (67.1)	tr*[1] (tr)	ND (ND)
C. annuum var. annuum cv. Red chilli	Mexico	0.13 (7.3)	0.78 (44.1)	0.84 (47.5)	0.02 (1.1)	ND (ND)
C. pubescens cv. Roccoto	Bolivia	1.01 (13.7)	2.79 (37.8)	3.59 (48.6)	ND (ND)	ND (ND)
C. annuum var. annuum cv. Karayatsubusa**	Kyoto (Japan)	0.32 (12.0)	1.34 (50.2)	0.96 (36.0)	0.05 (1.9)	ND (ND)
C. annuum var. annuum cv. Karayatsubusa***	Kyoto (Japan)	0.20 (7.5)	1.50 (56.2)	0.97 (36.3)	tr (tr)	ND (ND)

* Although the name of cultivar was uncertain, the *Capsicum* fruits purchased from three different areas in People's Republic of China originated from the same species. In spite of different capsaicinoid content, the compositions resembled each other.

** *C. annuum* var. *annuum* cv. Karayatsubusa was used to compare the reliability of the RP-8 HPTLC plates with mass chromatography[12].

*** Data obtained by mass chromatography. Approximately the same values were obtained for ** and *** except for NDC and HDC. The higher values for ** for NDC and HDC should be attributed to the tailing of excess amounts of DC and CAP. For detailed experimental conditions for the capsaicinoid extraction and HPTLC, see text.
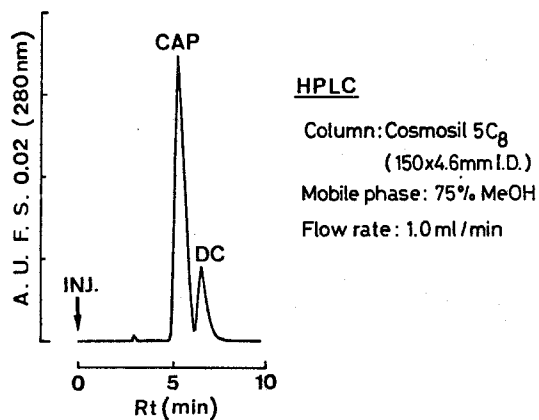
Fig.5. Chromatogram of capsaicin and dihydrocapsaicin
obtained by HPLC

HPLC

Clear separation of CAP and DC was achieved by eluting with 75% methanol(Fig.5). The retention times of CAP and DC were 5.0 min and 6.24 min, respectively. Calibration plots were obtained from 20 ng to 5 μg for both compounds with a detection-wave length of 280 nm.

In this thesis, the determination of the pungent principle of hot pepper was suitably done by the one-dimensional HPTLC or HPLC method.

REFERENCES

1. J.I.Suzuki, F.Tausing and R.E.Morse, Food Technol., 11, 100(1957).

2. J.J.Dicecco, J. Ass. Offic. Anal. Chem, 62, 998(1979).

3. V.S.Govindarajan and S.M.Ananthakrishna, Flavour Ind., 5, 176(1974).

4. D.S.Pankar and N.G.Magar, J. Chromatogr., 144, 149 (1977).

5. E.Kovacs, E.E.Kevei-Pichler and I.Lendvai, Acta Aliment., 6, 1(1977).

6. P.H.Todd,Jr., M.G.Bensinger and T.Biftu, J. Food Sci., 42, 660(1977).

7. J.Jurentisch, W.Kubelka and K.Jentsh, Sci. Pharm., 46, 307(1978).

8. J.Jurentisch, ibid., 47, 31(1979).

9. Y.Masada, K.Hashimoto, T.Inoue and M.Suzuki, J. Food Sci., 36, 858(1971).

10. K.R.Lee, T.Suzuki, M.Kobashi, K.Hasegawa and K.Iwai, J. Chromatogr., 123, 119(1976).

11. R.Rangoonwala and G.Seitz, Deut. Apoth.-Ztg., 110, 1946 (1970).

12. K.Iwai, K.R.Lee, M.Kobashi, T.Suzuki and S.Oka, Agric. Biol. Chem., 41, 1873(1977).

13. K.Iwai, T.Suzuki, H.Fujiwake and S.Oka, J. Chromatogr.,

172, 303(1979).

(i) T.Suzuki, T.Kawada and K.Iwai, <u>J. Chromatogr</u>., **198**, 217
(1980).

CHAPTER 2

GASTROINTESTINAL ABSORPTION AND MOVEMENT OF PUNGENT PRINCIPLE OF HOT PEPPER IN RATS [ii]

Monsereenusorn(1) reported that commercial "Capsaicin" (Sigma), a mixture of capsaicin(CAP) and dihydrocapsaicin(DC), was absorbed in everted sac of jejunum. However, Kim and Park(2) suggested that CAP and its analogues were poorly absorbed in gut. The degree and mechanism of absorption of CAP or its analogues in the gastrointestinal tract have not yet been elucidated.

In this chapter the author describes the gastrointestinal absorption and movement of CAP and DC in vivo and in situ rats.

EXPERIMENTAL

Materials

DC and 8-methyl nonanoic acid(Iso-$C_{10:0}$) used for the standard were synthesized chemically as reported previously(3). Commercial "capsaicin mixture", which was a mixture of CAP(85%) and DC(15%) as determined by using HPTLC(Chapter 1), was purchased from E.Merck(Darmstadt, G.F.R. Lot No. 95528). [nonanamide-6,7,9-^{3}H(N)]-DC and methoxy-inulin [methoxy-^{3}H] were obtained from the New Engrand Nuclear(Boston, U.S.A.). The radiochemical purity of [^{3}H]-DC was determined as 98% by thin-layer chromatography on Silica Gel 60 plate(E.Merck) using n-hexane-diethyl ether-acetic

acid(50:50:1,v/v). Hyamine 10X-OH was obtained from Nakarai Chemicals Co.(Kyoto). The scintillation fluid used was a "Triton X-100" emulsion system(4). All other chemicals used were of guaranteed reagent grade.

Gastrointestinal absorption of CAP and DC by rat in vivo

Male Wistar rats, fasted overnight but permitted free access to water, were used as experimental animals. Since we usually ingest the pugent principle of hot pepper as a mixture of CAP and its analogues with food, "capsaicin mixture(E.Merck)" was used in this experiment. The doses of CAP and DC used in this study were related to that usually ingested by rural Thai people(5). The rats(ca.220 g) were orally administered 3 mg of "capsaicin mix-ture" suspended in basal diet(casein 200 mg, soluble starch 350 mg sucrose 300 mg, salt mixture 40 mg and soybean oil 100 μl in saturated phenol red sol. 2 ml) by stomach tube. After various time intervals, the rats were killed by dislocation of the spinal column. The gastrointestinal tract was divided with pairs of ligatures into six segments (**stomach, duodenum, jejunum, ileum, cecum, and large intestine**). Their contents were washed with 3 times with 5 ml of 2 mM sodium deoxycholate. Phenol red was as a volume marker and its concentration was determined spectrophoto-metrically by the difference in absorbancy(560 nm) of pH 11.0 and 7.2. The CAP and DC present in tissue were extracted three times by 5 ml of chloroform-methanol(2:1,v/v) and dried with N_2.

Residual CAP and DC in intestinal contents were extracted three times by 5 ml of chloroform and dried with N_2. CAP and DC contents were determined by HPLC as described in Chapter 1.

Gastrointestinal absorption of CAP and DC in situ

Regional capacities for absorption were studied by the modified in situ loop method of Kimura et al.(6). The rats (200-250 g) were anethetized with sodium pentobarbital(55 mg/kg, i.p.), and the abdomen was cut open by a midline incision. Cardiac and pyloric orifices were ligated (**stomachal loop**), and the small intestine was divided into the following two loops by ligatures; the 10 cm length beginning 2 cm beyond the pyloric orifice (**jejunal loop**) and the 10 cm length terminating 2 cm from the cecum (**ileal loop**). Emulsions(1 ml) composed of 1 mM "capsaicin mixture", 0.3% Tween 20 in 0.9% saline for stomach, and 1 mM "capsaicin mixture", 5 mM sodium taurocholate and 5 mM monoolein in 0.15 M phosphate buffer(pH 6.3) for the small intestine were injected into these loops by syringe and left in situ for a given interval. Since these emulsions were homogeneous than suspension with feed in vivo, they are suited for the study of regional absorption capacities. When the incubation was over, the CAP and DC present in tissues and contents were extracted as discribed above. CAP and DC were determined by HPLC as Chapter 1. Since it was difficult to investigate directly the nonspecific adsorption of CAP and DC in gut, the adsorption was measured by the method of

Cotlove(7) using a nonabsorbable marker, [3H]-inulin.

Effect of metabolic inhibitors on [3H]-DC uptake

Initial uptake of DC with or without inhibitors was investigated using the ligated jejunal loops(5 cm length beginning 7 cm beyond the pyloric orifice). DC emulsion contained 1 mM [3H]-DC(specific activity, 1.0 mCi/mmol), 5 mM sodium taurocholate and 5 mM monoolein in 0.15 M phosphate buffer(pH 6.3). The metabolic inhibitors used were 1 mM 2,4-dinitrophenol(DNP) or 10 mM NaCN. My preliminary experiments have shown that the effect of metabolic inhibitors on DC uptake was unchanged at even higher concentrations(10 mM DNP, 100 mM NaCN) and/or longer exposure time(30 min), but the animals are hard to maintain alive for a longer period owing to high toxicity of DNP or NaCN. Consequently, the short time exposure method was used. The low temperature-treatment of jejunal loops was at 20°C (indirectly cooled by ice).

The [3H]-DC emulsion(400 μl) was injected into the loop preincubated with aqueous solution of inhibitors for 3 min. After the 3-min incubation, the infusate was rapidly washed out three times with 5 ml of 2 mM sodium deoxycholate. An aliquot of combined washes(200 μl) was transferred to a vial containing 5 ml of scintillation fruid. Radioactivity was measured with a Packard automatic Tri-Carb liquid scintillation spectrometer, Model 3255. Quenching was corrected for by the external standardization method

Appearance of [3H]-DC in mesenteric venous blood in situ

The modified in situ method of Kimura et al.(6) was employed. Male Wistar rats, weighing 250-300g, were fasted overnight. While the rats were under sodium pentobarbital anesthesia, their abdomens were cut open by midline incision. A loop (4 cm) of jejunum with a methenteric venous branch was was prepared. The mesenteric vein was cannulated after intra-venous instillation of heparinized 0.9% saline solution into the thigh vein, and then the emuision (400 µl) was injected into the loop. After [^{3}H]-DC was injected into the closed jejunal loop, the mesenteric venous blood was collected for 2-5 min intervals. [^{3}H]-DC emulsion was composed of 1 mM [^{3}H]-DC (specific activity, 1.0 mCi/mmol), 5 mM sodium taurocholate and 5 mM monoolein in 0.15 M phosphate buffer(pH 6.3). The blood volume in each fraction was measured with a syringe, and the blood flow-rate was expressed in ml per min. The radioactivity in blood was measured with a liquid scintillation spectrometer as described above. The blood suspention(125 µl) was solubilized with Hyamine (500 µl), then decolarized by H_2O_2(100 µl). The presence of radioactive metabolites was examined by HPTLC on pre-coated plates (cellulose, E.Merck), with a solvent system: 1-butanol-1.5 M NH$_4$OH(1:1,v/v,upper phase) and TLC(Silica Gel 60 plate, E.Merck) with a solvent system: n-hexane-diethyl ether-acetic acid(50:50:1, v/v).

Identification and quantitation of a radioactive metabolite of

[3H]-DC in portal blood

The mesenteric venous blood(500 μl) was hemolyzed by adding distilled water, then DC and its metabolites were extracted by ethyl acetate. The combined extracts were dried under a N_2 stream and dissolved in methanol(100 μl). It was ascertained that the aqueous phase contained no radioactivity. The [3H] metabolite of [3H]-DC was separated and identified by HPTLC and TLC as above. Appropriate bands were removed from the plates and transferred into counting vials.

Polyacrylamide gel disc electrophoresis

The 3.0 and 5.0% polyacrylamide separation gels(pH 9.4) were prepared by a modification of the method of Narayan et al.(8). Each fraction was stained with Sudan Black B. Radioassay was performed on 2 mm gel segments of stained gel after discoloration according to Moss and Ingram(9).

In vitro metabolism of DC in jejunal tissue

The isolated jejunums from male rats(ca. 220 g) fasted overnight were washed with 0.9% saline solution, minced into small pieces and homogenized with 5 volume of an isolation medium(0.1 M potassium phosphate buffer, pH 7.4, containing 10 mM 2-mercaptoethanol) in a teflon pestle homogenizer. The reaction mixture consisted of unlabelled DC(1.0 mM), Triton X-100(0.1%), neomycin sulfate(5 mg) and crude homogenate(5 mg of protein) in a final volume of 1.0 ml. The whole system was incubated for 2 hr at 37°C

The metabolites were extracted 3 times with 5 ml of chloroform-methanol(2:1,v/v). The combined chloroform layer was evaporated to dryness under a N_2 steam. The metabolites were first subjected to fractionation by TLC(Silica Gel 60, E.Merck) with n-butanol-acetic acid-water(60:10:50,v/v, upper phase). Metabolites were detected by iodine vapor, by spraying 0.1% 2,6-dichloroquinone chloroimide in methanol or by 0.2% ninhydrin in acetone. The zone corresponding to the metabolites were removed and extracted with methanol. The solvent was evaporated under a N_2 stream, and the residues were reacted with trimethylsilylated for GC-MS analysis of vanillylamine or methylated for GC analysis of Iso-$C_{10:0}$ fatty acid.

GC-MS analysis

The GC-MS of trimethylsilyl(TMS) derivatives of jejunal metabolites separated by TLC was carried out on a Shimadzu-LKB 9000 gas chromatograph-mass spectrometer. The separation column was a coiled glass column(2 m x 2 mm I.D.) packed with 3% Silicon SE-30 on Chromosorb W(60-80 mesh). The column temperature was programmed from 160-250 °C at a heating rate of 5°C/min. The carrier gas(helium) flow rate was at 30 ml/min.

GC analysis

The system for GC consisted of a Shimadzu GC-6AM equipped with FID, Shimadzu E1A microcomputer, a glass column(1.5 m x 3 mm I.D.) packed with 5% Silicon SE-30 on Chromosorb W(60-80 mesh).
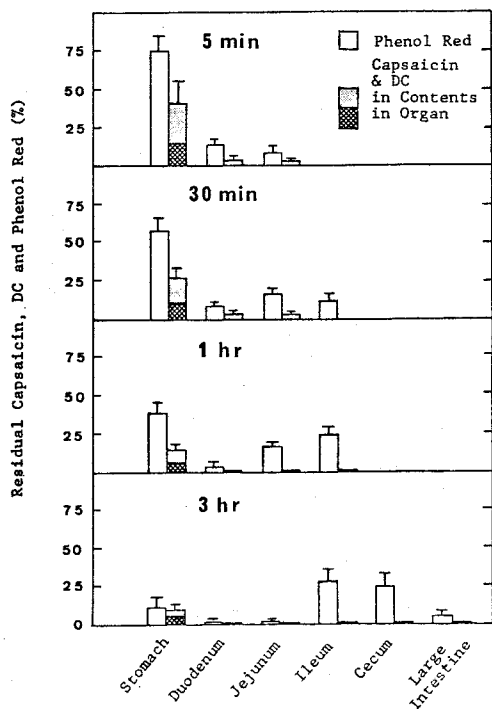
FIG. 1. The distribution of capsaicin, dihydrocapsaicin (DC), and phenol red in the gastrointestinal tract of rats at various intervals after po administration of 3 mg "capsaicin mixture (E. Merck)." Capsaicin and DC were removed from the gut in the same relative proportions as the Merck mixture used. Each value represents $\bar{x} \pm SD$ of at least five rats.
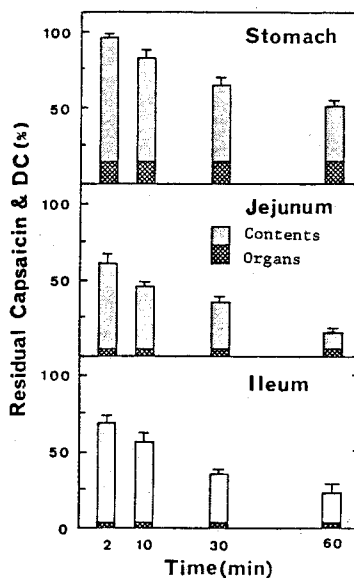
FIG. 2. Disappearance of capsaicin and dihydrocapsaicin from the ligated loops of stomach (top), jejunum (center), and ileum (bottom) *in situ*. Stomach loops were infused with 1 mM "capsaicin mixture (E. Merck)," 0.3% Tween 20 in 0.9% saline (1 ml), and jejunum (10 cm) or ileum (10 cm) was incubated with 1 mM "capsaicin mixture," 5 mM sodium taurocholate, 5 mM monoolein in 0.15 M phosphate buffer (pH 6.3, 1 ml), from 2 to 6 min. Capsaicin and DC were removed from the gut in the same relative proportions as present in the Merck mixture used. Each value represents $\bar{x} \pm SD$ of at least five rats.

The temperature was programmed from 90–180 °C at a heating rate of 4 °C/min, and N_2 was the carrier gas flow rate at 50 ml/min.

RESULTS

In vivo absorption

The time course of CAP and DC absorption from the gastrointestinal tract of rats is shown in Fig.1. Of the dose, 50, 30, 20 and 15% remained in the whole gastrointestinal tracts at 5 min, 30 min, 1 hr and 3 hr after administration, respectively.

A significant amount of CAP and DC disappereared from the stomach during the first 5 min. After 5 min the pungencies were mainly absorbed in superior portion of small intestine(duodenum and jejunum). CAP and DC were absorbed in the same relative proportion as present in the Merck mixture. No metabolite of CAP or DC was detected in the gut contents. The amount of CAP and DC in feces was less than 10% of the administered dose after 48 hr(data not shown).

In situ absorption

The rate of absoption of CAP and DC from stomach and small intestine can be seen from the results presented in Fig.2. Inulin spaces in stomach, jejunum and ileum were 1.5, 4.7 and 3.8% of the wet tissue weight, respectively. Consequently, residual CAP and DC contents were corrected by the inulin spaces. CAP and DC were absorbed in the same ralative proportions as in the Merck mixture used. Within 60 min of administration into stomach, jejunum and ileum, approximately 50, 80 and 70% of the dose had disappeared from the lumen, respectively, indicating that CAP and DC were absorbed more readily from jejunum and ileum than stomach. Since no metabolite of CAP or DC was detected in the gut contents, hydrolysis by the gut contents(acidity or bacteria) should have not taken place.

Effect of metabolic inhibitors

To reveal whether the absorption of CAP and its analogues are

FIG. 3. The appearance of [³H]dihydrocapsaicin and its metabolites in mesenteric venous blood from ligated jejunal loops. The loops (4 cm) were infused with 1 mM [³H]dihydrocapsaicin, 5 mM sodium taurocholate, and 5 mM monoolein in 0.15 M phosphate buffer (pH 6.3, 400 μl). Points are presented as cumulative radioactivity (dpm) while the blood flow rate in ml/min is represented by straight lines. Each value represents $\bar{x} \pm$ SD of four rats.

due to active process or not, the effect of metabolic inhibitors on the uptake of [³H]-DC was examined by using short time exposure method. [³H]-DC was taken up at 2.18 ± 0.12%(mean ± S.D.) of the dose per min per 5 cm length of loop in the control experiment. No significant reduction in uptake was observed in the incubation systems in which DNP or NaCN was added to the emulsion, or when the loops were kept at 20°C(Table 1).

Route of absorption

The appearence of ³H was found in the portal blood, and the activity in proportion to the incubation time as shown in Fig.3;
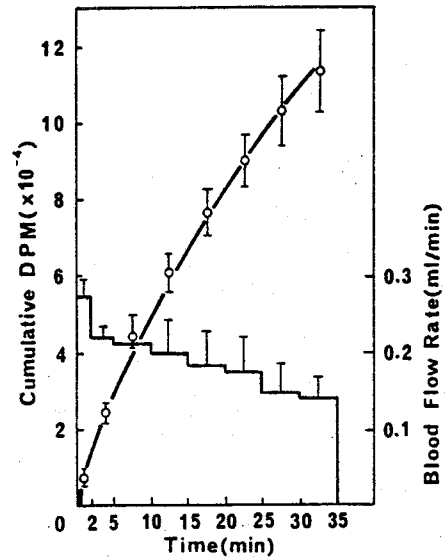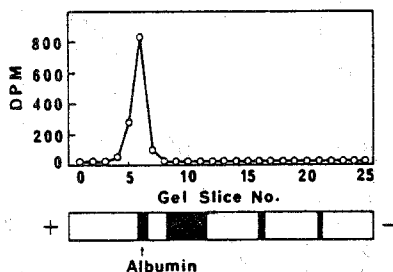
FIG. 4. Association of radioactive substances with serum proteins of mesenteric venous blood. Serum (30 μl, 5- to 10-min fraction) was subjected to the separation of proteins by disk gel electrophoresis. Serum proteins were separated into four fractions (albumin, α, β, and pre-β lipoprotein from left to right). Each value represents the mean of three experiments.

TABLE 2

METABOLISM OF DIHYDROCAPSAICIN IN THE INTESTINAL TISSUE DURING ABSORPTION[a]

Fraction (min)	% of total radioactivity[b]	
	DC	Iso-$C_{10:0}$
0–2	82.5 ± 1.6	17.5 ± 1.6
5–10	87.3 ± 2.4	12.7 ± 2.4
15–20	88.3 ± 3.3	11.7 ± 3.3
25–30	83.0 ± 4.9	17.0 ± 4.9

[a] 1 mM [^{3}H]DC emulsion (0.4 ml) was injected into the ligated jejunal loop (4 cm) and the radioactive substances were extracted from the mesenteric vessel blood (500 μl) in each fraction. Each radioactive substance was separated by thin-layer chromatography (silica gel G).
[b] Each value is $\bar{x}$ ± SD from four rats.

twenty five % of the radioactivity was recovered in the mesenteric vessel blood from jejunum(4 cm length of loop) within 35 min in situ. When the portal serum proteins were separated by electro-phoresis, radioactivities of DC and Iso-$C_{10:0}$ were found to be associated with the albumin fraction(Fig.4). No metabolite of [^{3}H]-DC was detected in the closed loop infusate and mesenteric lymph collected by lymphatic cannulation had scarcely any radio-activity within the first 2 hr(data not shown).

Radioactive metabolite of [^{3}H]-DC in the mesenteric venous blood

To study the metabolism of CAP and its analogues in the intestinal tissue, the radioactive substances were extracted from the mesenteric venous blood after the administration of [^{3}H]-DC into the ligated loops in situ and identified by HPTLC and TLC. Radioactive materials present in the blood were identical to
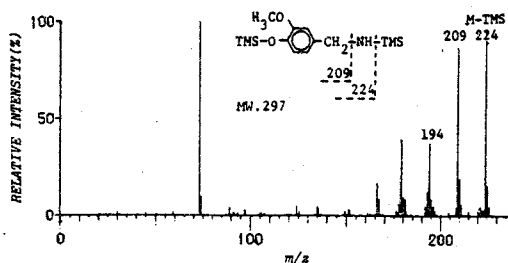
FIG. 5. Mass spectrum of trimethylsilyl derivative of phenolic metabolite formed from dihydrocapsaicin by cell-free extracts of rat jejunal tissue.

parent $[^3H]$-DC and $[^3H]$-Iso-$C_{10:0}$. As shown in Table 2, after the administration of $[^3H]$-DC, approximately 85% of the blood radioactivity was detected as $[^3H]$-DC, while about 15% was Iso-$C_{10:0}$ throughout the incubation period. No radioactive metabolite except Iso-$C_{10:0}$ was found. Since no metabolite of $[^3H]$-DC was detected in jejunal loop infusate, no metabolism of DC presumably took place in the gut contents.

In vitro metabolism of DC in jejunal tissue

After incubating DC with cell-free extracts of jejunal tissue a phenolic metabolite was located on the TLC plate. The spot having Rf 0.41 should presumably be identical to vanillylamine as judged from the Rf value and color reactions. The metabolite separated by TLC was further subjected to GC-MS after trimethyl-silylation. The spectrum pattern of the TMS derivative was iden-tical to that of vanillylamine(Fig.5). The molecular ion was not observed in the mass spectrum. Detection of vanillylamine in a

reaction mixture means that DC is enzymically hydrolyzed at its acid-amide bond, and releases Iso-$C_{10:0}$. Iso-$C_{10:0}$ released in the reaction mixture was detected by GC(data not shown). No metabolite was found in a reaction mixture with boiled cell-free extracts.

DISCUSSION

An interesting report on the acute toxicity in mice was recently presented by Glinsukon et al.(9) in which the toxicity after orally administered CAP(Sigma Chemical Co., a mixture of CAP and DC) was far less than that of i.v., i.m. or i.p. Reports on the gastrointestinal absorption of CAP and its analogues have been few(1,2).

My present studies using rats in vivo showed that gastro-intestinal absorption of "capsaicin mixture(E.Merck)", especially in the small intestine, took place during 3 hr after oral admini-stration. Data from reliable in situ experiments also indicated that CAP and its analogues were mainly absorbed at the superior portion of the small intestine. It was also noticed in situ expe-riments that the disappearence rate of CAP and DC from the jejunal loop(2.0% of the dose/min/cm length of loop, Fig.2) was nearly equal to the appearence-rate of radioactivity in the blood stream (1.9% of the dose/min/cm length of loop, Fig.3) 30 min after the injection.

Levine et al.(10) indicated that intestinal sacs of rats progressively lose structual integrity. Therefore, experimental conditions as near to physiological as possible are needed such as in situ condition(6). My present results in vivo and in situ are not in conflict with the conclusion from the in vivo experiment with the everted sac(1). On the other hand, Kim and Park(2) reported from their experiments in vivo that the absorption of CAP administered orally was poor in the gastrointestinal tract of rabbits, based on the assumption that CAP absorbed from the tract should be excreted in urine without any biotransformation. However, I have recently found that the major part of DC administered orally to rats is metabolized rapidly and excreted in urine(see Chapter 3).

Monsereenusorn(1) proposed a passive diffusion mechanism from experiments using the everted sac of jejunum without examining the effects of metabolic inhibitors. In the present experiments, the effects of metabolic inhibitors on the uptake of [3H] DC were examined. Neither DNP, an uncoupler of the oxidative phosphorylation, nor NaCN, an inhibitor of the respiratory chain, showed effects on the uptake of [3H]-DC in jejunum in situ. If the absorption of [3H]-DC takes place by an active transport mechanism, the uptake of [3H]-DC should have been considerably depressed in the presence of inhibitors. Furthermore, no change was observed on the [3H]-DC uptake in the jejunum at low temper-

ature(20°C). This also indicates that CAP and its analogues are absorbed by a nonactive process.

In the present study, I have also found that $[^3H]$-DC was not transported into the mesenteric lymph but to the mesenteric vein(Fig.3). CAP and its analogues are capable of causing and intensifying damage to duodenal absorptive cells(11,12). However, no metabolite of CAP or DC was detected in the contents or infusate of in vivo and in situ experiments. Consequently,it is acceptable that DC is partly hydrolyzed to vanillylamine and Iso-$C_{10:0}$ fatty acid when DC passes through the epithelial cells of the jejunum(Table 2); CAP and its analogues partly receive a first-pass effect, metabolism of compounds during its first absorption(not via an enterohepatic circulation) in the tract. This possibility was strongly supported by the detection of DC-hydrolyzing enzyme activity in jejunal tissue in vivo. DC and its metabolite(Iso-$C_{10:0}$) were mainly associated with the serum albumin in the mesenteric venous blood and then were transported into the body.

It is interesting to know the mechanism of lower toxicity of CAP and its analogues after oral dose than the others. The present study shows that CAP and DC are surely absorbed in the gastro-intestinal tract of rat, then they receive a first-pass effect in the tract. This effect may be partly contributed to lower toxicity of the pungency administered orally.

References

1. Y.Monsereenusorn, <u>Toxicol. Appl. Pharmacol.</u>, **53**, 134 (1980).

2. N.D.Kim and C.Y.Park, <u>J. Pharm. Soc. Korea.</u>, **25**, 101 (1981).

3. K.Iwai, K.R.Lee, M.Kobashi, T.Suzuki, and S.Oka, <u>Agric. Biol. Chem.</u>, **42**, 202(1978).

4. P.H.Williams, <u>Int. J. Appl. Radiat. Isotopes.</u>, **19**, 377 (1968).

5. Interdepartment Committee on Nutrition for National Defense. <u>Nutrition Survey-The Kingdom of Thailand.</u> pp 57, U.S. Government Printing Office, Washington, D.C. (1962).

6. S.Kimura, M.Totani, and T.Murachi, <u>Japan J. Exp. Med.</u>, **43** 163(1973).

7. E.Cotlove, <u>Amer. J. Physiol.</u>, **176**, 393(1954).

8. K.A.Narayan, H.L.Creinin., and F.A.Kummerow, <u>J. Lipid Res.</u> **7**, 150(1966).

9. T.Glinsukon, V.Stitmunnaithum, C.Toskulkao, T.Burannawuti, and V.Tangkrisanavinont, <u>Toxicon.</u>, **18**, 215(1980).

10. R.R.Levine, W.F.Mcnary, P.J.Kornguth, and R.Leblanc, <u>Eur. J. Pharmacol.</u>, **9**, 211(1970).

11. W.Nopanitaya, <u>Digestive Diseases</u>, **19**, 439(1974).

12. W.Nopanitaya and Y.Nye, <u>Toxicol. Appl. Pharmacol.</u>, **30**, 149(1974).

(ii). T.Kawada, T.Suzuki, M.Takahashi, and K.Iwai, <u>Toxicol.</u>

<u>Appl.</u> <u>Pharmacol.</u> in press.

CHAPTER 3

IN VIVO AND IN VITRO METABOLISM OF PUNGENT PRINCIPLE OF HOT PEPPER IN RATS

In chapter 2, it was revealed that CAP and DC are readily transported through the gastrointestinal tract by nonactive transport into the portal vein and partly metabolized during absorption Thinking about the fact CAP and its analogues have been taken as an ingredient of spices into our bodies, it is important to investigate the biotransformation and the detoxication of CAP and its analogues. However, the paper on the metabolism of CAP and its analogues in animals has not been reported except the study using microsomes of rat liver(1).

In this chapter, the author described the biotransformation and detoxication of DC in vivo and in vitro revealed by novel chromatographic procedures for the analyses of urinary and hepatic metabolites of DC.

EXPERIMENTAL

Materials

DC and 8-methyl nonanoic acid(Iso-$C_{10:0}$) used for the standard were synthesized chemically as reported previously(2). Vanillin, vanillylalcohol and vanillic acid were purchased from Tokyo Kasei Kogyo(Tokyo). Vanillylamine hydrochloride was

- 33 -

obtained from Aldorich Chemical Company(Milwaukee, U.S.A.).
[nonanamide-6,7,9-^{3}H(N)] dihydrocapsaicin ([^{3}H]-DC) was
obtained from New England Nuclear(Boston, U.S.A.). Beta-glucuro-
nidase(Type H-I) was purchased from Sigma(St. Louis, U.S.A.). All
other chemicals used were of guaranteed reagent grade.

Treatment of animals

   Male Wistar rats weighing 280-300 g were used in experiments
after 3 days feeding with purified diet(Oriental Yeast Co., Tokyo)
containing(by wt %): 20% casein, 50% starch, 15% sucrose, 8% soy-
bean oil, 4% salt mixture, 1% vitamin mixture and 2% cellulose.
The rats were fed the diet and water <u>ad lib</u>. Synthetic DC(6.0
mg) was administered by a stomach tube as a suspension in olive
oil(200 µl). Urine and feces were separately collected every 24
hr for 2 days. These samples were stored at - 20°C until use.
Five rats were used in each experiment.

Extraction of urinary metabolites

   The urine sample, after thawing and filtration through
defatted cotton, was diluted with water to 20 ml and divided into
two equal portions(10 ml) were acidified to pH 2.0 with 4 N HCl
and extracted 3 times with each 6 ml of ethyl acetate. The combi-
ned organic phase was dried over anhyd. Na_2SO_4, and the dried
organic phase containing metabolites was evaporated to dryness
under a N_2 stream. These samples containing free metabolites
were dissoleved in methanol(200 µl) [Free metabolites extracted

with ethyl acetate]. Further, the ramaining aqueous portion was adjusted to pH 12.0 with 4 N NaOH, and vanillylamine was extracted 3 times with each 6 ml of chloroform-methanol(2:1, v/v). The combined organic phase was then dried over anhyd. Na_2SO_4, and the sample was evaporated under a N_2 stream. These samples were dissolved in chloroform-methanol(2:1, v/v, 200 μl) [Free metabolites extracted with chloroform-methanol].

The other urine(10 ml) was adjusted to pH 5.0 with saturated $NaHCO_3$ solution and treated with beta-glucuronidase(ca. 5000 fishman units) at 37°C for 10 hr after addition of 0.2 M acetate buffer(pH 5.0, 2 ml) and neomycin sulfate(0.5 mg). This sample containing free and glucuronide metabolites was extracted with ethyl acetate, then chloroform-methanol(2:1, v/v) in a similar manner as above [Total metabolites extracted with ethyl acetate, and Total metabolites extracted with chloroform-methanol].

Extraction of DC from feces

Total feces were powdered in a mortar, then used for the extraction of DC with chloroform-methanol(2:1, v/v). Extracts were purified by the use of SEP-PAK C_{18} cartridge(Waters Associates, MA, U.S.A.) and methanol as a elution solvent. Eluates were evaporated to dryness under air stream. These sample are dissolved in chloroform-methanol(2:1, v/v, 400 μl).

Preparation of cell-free extracts of rat liver

Unless otherwise indicated, all operations were carried out

at 0-4°C. The isolated liver from male Wiatar rats(250-290 g) fasted overnight was minced into small pieces and homogenized with 9 vol. of 50 mM potassium phosphate buffer(pH 7.0) in a teflon pestle homogenizer. After large tissue fragments and nuclear were removed by centrifugation at 600 x g for 10 min, the supernatant was brought to 80% saturation with solid ammonium sulfate. The mixture was allowed to stand for at least 1 hr, then centrifuged at 10,000 x g for 20 min. The resulting precipitate dissolved in a small volume of the isolation buffer, dialyzed against the same buffer. The supernatant containing mainly mitochondria, microsome and soluble fraction was used as the enzyme source. The enzyme preparation was kept at - 20°C until use. The enzyme activity in the form of 80% ammonium sulfate suspension was not lost for at least 1 month. Protein concentration was determined by the method of Lowry et al.(3).

DC metabolism by cell-free extracts of rat liver

The reaction mixture contained 50 mM potassium phosphate buffer(pH 7.0), 1 mM synthetic DC, 0.05% Tween 80 and the enzyme solution(6.0 mg of protein) in a final volume of 1.0 ml. The whole system was incubated at 37°C for 3 hr, then the reaction was terminated by the addition of 2 ml of chloroform-methanol(2:1, v/v). The mixture was vigorously shaken, followed by centrifuged at 600 x g for 10 min. After an additional extraction of metabolites with chloroform-methanol(2:1, v/v), the combined chloroform

layer was evaporated to dryness under a N_2 stream. The meta-
bolites were dissolved in chloroform-methanol(2:1, v/v, 200 μl),
then subjected to TLC, GC, and GC-MS analyses for the identifi-
cation and quantitation of metabolites.

DC-hydrolyzing enzyme activity in various organs

DC-hydrolyzing enzyme activity in various organs was assayed
by using radioisotopic technique described below. Each organ was
homogenized in 5 vol. of 0.1 M potassium phosphate buffer(pH 7.4)
at 0-4°C. Reaction mixture consisted of 3 mM $[^3H]$-DC(sp. act.,
0.81 μCi/nmol), 10 mM potassium phosphate buffer(pH 7.4), 0.1%
Triton X-100 and crude homogenate(4.0 mg of protein) in a final
volume of 1.0 ml. The whole system was incubated at 37°C for 1 hr.
The reaction was terminated by addition of 200 μl of conc.
H_3PO_4. For the extraction of metabolites, diethyl ether(2 ml)
was added to the incubation mixture, which was then shaken
thoroughly and centrifuged at 600 x g for 10 min. After an addi-
tional extraction of metabolites with ether, the combined ether
layer was evaporated to dryness under a N_2 stream. The metabo-
lites were dissolved in methanol(200 μl). The methanol solution
was used for the measurment of radioactivity of metabolite(Iso-
$C_{10:0}$). The radioactivity of Iso-$C_{10:0}$ was analyzed by TLC
using a Silica Gel 60 TLC plate(E. Merck, 250 μ thick) developed
with n-hexane-diethyl ether-acetic acid(50:50:1, v/v). Iso-$C_{10:0}$
was detected by iodine vapor. The Rf value for authentic Iso-

$C_{10:0}$ was 0.57. The region corresponding to $Iso-C_{10:0}$ was scraped off and transferred into a vial containing 5 ml of scintillation fluid(4). Radioactivity was measured as discribed in chapter 2.

TLC

Urinary metabolites were chromatographed on 20 x 20 cm precoated Silica Gel 60 TLC plate(E. Merck), using chloroform-acetic acid(90:10, v/v)[Solvent system a] and benzene-ethyl acetate-acetic acid(70:30:1, v/v)[Solvent system b] as developers.

Hepatic metabolites were first subjected to two dimentional TLC on Silica Gel 60 plate developed with 1-butanol-acetic acid-water(60:10:50, v/v, upper phase) as the first solvent system [Solvent system c], air-dried, then developed further with chloroform-ethyl acetate(90:10, v/v) as the second solvent system [Solvent system d]. The zone corresponding to the hepatic metabolites were scraped off and extracted with methanol. The solvent was evaporated under a N_2 stream, and the residues were trimethylsilylated for the GC-MS analysis(5).

The metabolites on TLC plate were detected by iodine vapor, by 0.1% 2,6-dichloroquinone chloroimide in methanol or by 0.4% 2,4-dinitrophenyl hydrazine in 2 N HCl.

HPLC

The system for HPLC consisted as described in chapter 1. The mobile phase used for separating DC and vanillylamine was methanol-water(75:25, v/v) containing 0.004% SDS(w/v). The mobile phase used for the analyses of vanillylalcohol, vanillic acid, and vanillin was methanol-water-acetic acid(25:74:1, v/v). The flowrate was kept at 1.0 ml/min. Eluates of urinary metabolites of DC were collected at the fraction vent of the LC-3A, then the solvent was evaporated under a N_2 stream, and residues were trimethylsilylated for the GC-MS analysis(5).

GC

Iso-$C_{10:0}$ released from DC by the cell-free extracts of liver was methylated by dimethylformamide dimethylacetal(6),then determined by GC using n-$C_{11:0}$ as an internal standard. The system for GC consisted of a Shimadzu GC-6AM equipped with FID, a glass column(1.5 m x 3 mm I.D.) packed with 5% Silicon SE-30 on Chromosorb W(60-80 mesh). The temperature was programmed from 90-180°C at an increase rate of 4°C/min, and nitrogen used as the carrier gas was kept at 50 ml/min.

GC-MS

The GC-MS of TMS derivatives of urinary and hepatic metabolites separated by HPLC or TLC was carried out on a Shimadzu-LKB 9000 gas chromatography-mass spectrometry. The separation column used was a coiled glass column(2 m x 2 mm I.D.) packed with 3% OV-

17 on Chromosorb W for TMS-vanillin, TMS-vanillylalcohol and TMS-vanillic acid or 3% Silicon SE-30 on Chromosorb W for TMS-DC and TMS-vanillylamine analysis. The column temperature used for OV-17 and SE-30 was programmed from 120-190°C at an increase rate of 5°C/min and 160-250°C at an increase rate 5°C/min, respectively. The carrier gas(helium) flow rate was kept at 30 ml/min.

RESULTS

TLC of urinary metabolites

The results of TLC analysis of the total urinary metabolites extracted with ethyl acetate are summarized in Table 1. By exposing to iodine vapor three spots were newly detected besides DC after development with two different solvent systems. The spots having (1) Rf 0.23, 0.41 and 0.49 which were obtained with solvent system a, and (2) Rf 0.22, 0.32 and 0.47 with solvent system b were represented M-I, M-II and M-III, respectively.

M-I, M-II and M-III showed identical Rf values and color reaction to those of vanillylalcohol, vanillic acid and vanillin, respectively.

HPLC of urinary metabolites

(1) Clear separation of vanillylalcohol, vanillic acid and vanillin was achieved by employing a mixture of methanol-water-acetic acid(25:74:1, v/v) as the developer on a reversed-phase column. The retention times and detection limits were determined
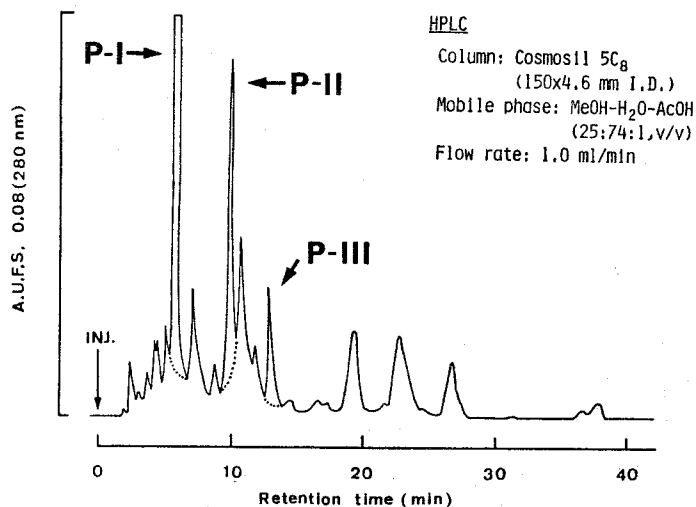
Fig.1. High-performance liquid chromatogram of rat urine specimens [Total metabolites extracted with ethyl acetate] 0-24 hr after oral administration of dihydrocapsaicin. control urine specimens (---)

Table 1. Rf Values and Colors of Dihydrocapsaicin and Its Urinary Metabolites on TLC Plate[*]

	Rf values in solvent		Color reaction	
	a	b	A	B
M-I	0.23	0.22	blue	-
M-II	0.41	0.32	blue	-
M-III	0.49	0.47	-	yellow
Vanillyl alcohol	0.23	0.22	blue	-
Vanillic acid	0.41	0.32	blue	-
Vanillin	0.49	0.47	-	yellow
Dihydrocapsaicin	0.43	0.47	blue	-

[*] Total metabolites extracted with ethyl acetate were subjected to the analysis of Silica Gel 60. Solvent system a, chloroform-acetic acid (90:10, v/v); b, benzene-ethyl acetate-acetic acid (70:30:1, v/v). Color reaction A, 0.1% 2,6-dichloroquinone chloroimide in methanol, B, 0.4% 2,4-dinitrophenyl hydrazine in 2 N HCl
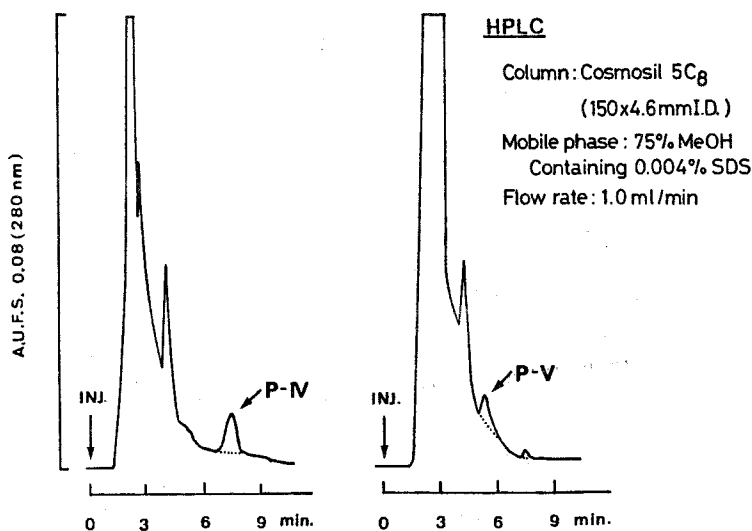
Fig.2. High-performance liquid chromatograms of rat urine specimens [Total
metabolites extracted with ethyl acetate (left) and with chloroform-methanol
(2:1, v/v)(right)] 0-24 hr after oral administration of dihydrocapsaicin.
control urine specimens (---)

for the phenolic compounds using the standard mixture; vanillyl-
alcohol(5.2 min, 15 ng), vanillic acid(9.9 min, 10 ng), and
vanillin(12.6 min, 10 ng).

Ethyl acetate extracts of urinary metabolites prepared after
oral administration of DC [Total metabolites extracted with ethyl
actate] was chromatographed as shown in Fig.1. Control urine
specimen is drown by dashed lines. Three peaks(P-I, P-II and P-
III) were newly detected in the sample after DC administration.
Retention times of P-I, P-II and P-III were identical to those of
vanillylalcohol, vanillic acid and vanillin, respectively.

(2) Clear separation of DC and vanillylamine was achieved by
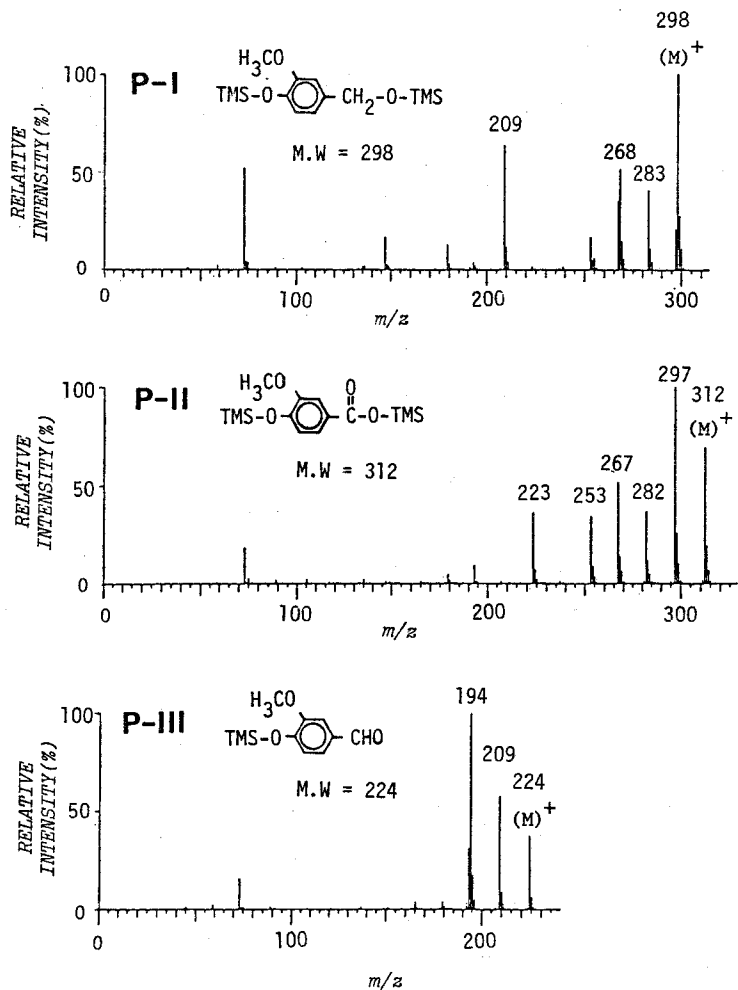eluting with a mixture of methanol-water(75:25, v/v) containing

Fig.3. Mass spectra of trimethylsilyl derivatives of dihydrocapsaicin

metabolites, P-I (above), P-II (centre) and P-III (below) separated by HPLC

0.004% SDS(w/v). The retention times of vanillylamine and DC were

5.4 and 7.5 min, respectively. The detection limits of both

compounds were 20 ng. Chromatograms of urinary metabolites eluted

by this solvent are shown in Fig.2. Dashed lines are control

urine specimen. Two peaks(P-IV and P-V) were newly detected in

the sample after DC administration. Retention times of P-IV and

Table 2. Distribution of Urinary Metabolites in Rats[*]

Metabolite	% Dose in			
	0 – 24 hr		24 – 48 hr	
	Free	Glucuronide	Free	Glucuronide
Dihydrocapsaicin	0.9 ± 0.5[**]	4.0 ± 0.6	1.4 ± 0.9	2.4 ± 0.5
Vanillylamine	0.9 ± 0.3	1.4 ± 0.4	1.3 ± 0.3	1.3 ± 0.4
Vanillyl alcohol	tr	27.5 ± 5.6	tr	10.1 ± 4.0
Vanillic acid	5.0 ± 1.4	8.8 ± 2.5	2.0 ± 0.4	3.4 ± 0.6
Vanillin	2.5 ± 0.9	1.3 ± 0.3	0.5 ± 0.3	0.3 ± 0.1

[*] Determination was carried out by the HPLC method as described in the text.

[**] Each value is the mean $\pm$ S.D. from five rats. tr; $< 0.1\%$

P-V were identical to those of DC and vanillylamine, respectively.

GC-MS of urinary metabolites of DC

Figure 3 shows the mass spectra of TMS derivatives of P-I, P-II and P-III fractionated by HPLC. The molecular and major fragment ions of P-I were m/z=298 $(M)^+$, m/z=283 $(M-TMS)^+$ and/or $(M-CH_3)^+$, m/z=268 $(M-TMS-CH_3)^+$ and/or $(M-2TMS)^+$. Those of P-II and P-III were m/z=312 $(M)^+$, m/z=297 $(M-TMS)^+$ and/or $(M-CH_3)^+$, m/z=282 $(M-TMS-CH_3)^+$ and/or $(M-2TMS)^+$, and m/z=224 $(M)^+$, m/z=209 $(M-TMS)^+$ and/or $(M-CH_3)^+$, m/z=194 $(M-TMS-CH_3)^+$, respectively. The spectral patterns of TMS derivatives of P-I, P-II and P-III were identical to those TMS derivatives of authentic vanillylalcohol, vanillic acid and vanillin, respectively. The mass spectra of TMS-derivatives of

P-V were identified to those of TMS-DC(5) and TMS-vanillylamine in chapter 2, respectively.

Quantitation of DC and its metabolites by HPLC

Table 2 summarizes distribution of DC metabolites in urine from rats determined by HPLC. About 75% of the original dose was excreted as unchanged DC and its metabolites into the urine within 48 hr. Total sum of free and glucuronide conjugates in urine after 48 hr were 14.5 and 60.4% of total dose, respectively. Major metabolites were the glucuronide of vanillylalcohol(37.6% of dose) that of vanillic acid(12.2%) and free vanillic acid(7.0%).

Chloroform-methanol(2:1, v/v) extracts of feces prepared after oral administration of DC contained 4.0 ± 0.5%(mean ± S.D., n=5) total dose of DC as parent DC for 0-24 hr sample and 5.9 ± 0.6%(n=5) for 24-48 hr sample.

Total recoveries of DC and its metabolites on the extraction procedures were in the range of 84-95%.

In vitro metabolism of DC by using cell-free extracts of rat liver

Incubating DC with cell-free extracts of rat liver, two phenolic metabolites were located on the TLC plate(Table 3). The spots having (1) Rf 0.41 and 0.82 with solvent system c, (2) Rf 0.03 and 0.32 with solvent system d were named M-IV and M-V, respectively. M-IV and M-V showed identical Rf values and color reactions to those of vanillylamine and vanillin, respectively.

Table 3. Rf Values and Colors of Dihydrocapsaicin Metabolites by Cell-free Extracts of Rat Liver on TLC Plate

	Rf values in solvent [*]		Color reaction [**]		
	c	d	A	B	C
M-IV	0.41	0.03	blue	–	yellow
M-V	0.82	0.32	–	yellow	–
Vanillylamine	0.41	0.03	blue	–	yellow
Vanillin	0.82	0.32	–	yellow	–

[*] Solvent system c, n-butanol-actic acid-water (40:10:50, v/v, upper phase); d, chloroform-ethyl acetate (90:10, v/v)

[**] Color reaction A, 0.1% 2,6-dichloroquinone chloroimide in methanol; B, 0.4% 2,4-dinitrophenyl hydrazine in 2 N HCl C, 0.25% ninhydrin in acetone

M-IV and M-V separated by TLC were further subjected to GC-MS after trimethylsilylation. The mass spectral patterns of TMS derivatives of M-IV [m/z=379 $(M)^+$, m/z=364 $(M-TMS)^+$ and/or $(M-CH_3)^+$] and M-V [m/z=224 $(M)^+$, m/z=209 $(M-TMS)^+$ and/or $(M-CH_3)^+$] were identical to those of P-V or vanillylamine in chapter 2, and P-III or vanillin(Fig. 3, bottom). Detection of vanillylamine in a reaction mixture means that DC is enzymically hydrolyzed at its acid-amide bond. The rate of Iso-$C_{10:0}$ relea-sed from DC at pH 7.0, catalyzed by the crude enzyme preparation, was linear for 3 hr(data not shown). No metabolite was detected in the incubation mixture with boiled cell-free extracts.

Distribution of DC-hydrolyzing enzyme activity in various organs

DC-hydrolyzing enzyme activity was found in various organs of

Table 4. Distribution of dihydrocapsaicin-hydrolyzing enzyme activity in various organs of rat

Organs	Enzyme activity (munits[*])	Specific activity (munits/mg protein)
Liver	304.2 $\pm$ 36.6[**]	5.74 $\pm$ 0.69
Kidney	100.2 $\pm$ 30.6	1.67 $\pm$ 0.51
Lung	66.6 $\pm$ 20.0	1.80 $\pm$ 0.54
Small intestine	64.4 $\pm$ 39.2	1.61 $\pm$ 0.98
Stomach	40.7 $\pm$ 24.7	2.14 $\pm$ 1.30
Brain	37.6 $\pm$ 4.4	1.88 $\pm$ 0.22
Thigh muscle	18.5 $\pm$ 12.5	0.43 $\pm$ 0.29
Heart	13.9 $\pm$ 4.0	0.73 $\pm$ 0.21

[*] One unit is defined as the release of one μmol of iso-$C_{10:0}$ per hr under the following assay conditions; the reaction mixture containing [^{3}H]-dihydrocapsaicin(3 mM), Triton X-100 (0.1%, w/w), potassium phosphate buffer, pH 7.4, (50 mM) and enzyme preparation in a final volume 1.0 ml was incubated at 37 °C for 1 hr under shaking at 60 r.p.m.

[**] Each value is the mean $\pm$ S.D. from four rats.

rat: liver, kidney, lung, intestine, stomach, brain, thigh muscle and heart(Table 4). The activity was mainly located and highest in liver. Therefore, it is suggested that liver is a main metabolic organ of pungent principle of hot pepper.

DISCUSSION

Nagy(7) suggested that the toxicity of CAP and its analogues had not been ascribed to any one facet of its actions but might be due to its precipitation of respiratory failure, bradycardia, and hypotension. The degree of acute toxicity of CAP and

its analogues was different by the route of administration in mouse: i.v. LD_{50} 0.56 mg/kg, i.p. LD_{50} 7.65 mg/kg, s.c LD_{50} 9.00 mg/kg, intragastric LD_{50} 190 mg/kg(8). The author described that CAP and DC were readily transported through the gastrointestinel tract by nonactive process to the portal vein, and hydrolyzed partly during absorption(see chaper 2). Consequently, it is important to investigate the biotransformation and detoxication of CAP and its analogues.

From the results of the present experiments in vivo and in vitro using rats, it was proved that DC was biotransformed to vanillylamine, vanillylalcohol, vanillic acid, vanillin and their glucuronide conjugates. It is speculated that DC absorbed into the portal vein is first hydrolyzed to vanillylamine and Iso-$C_{10:0}$ mainly in liver, then the former was transformed to vanillin after deamination. Subsequently, the aromatic aldehyde, vanillin, would be followed to major transformation pathways: oxidation and reduction.

Lee and Kumar(1) reported that CAP and its analogues were hydroxylated to N-(4,5-dihydroxyl-3-methoxybenzyl)-acylamide by a microsomal mixed-function oxidase in a sodium phenobarbital treated rat liver. However, under my experimental condition using either cell-free extracts(partly purified) or homogenate of rat liver, such a metabolite was not detected. Generally, administration of various chemical substances increases the activity of
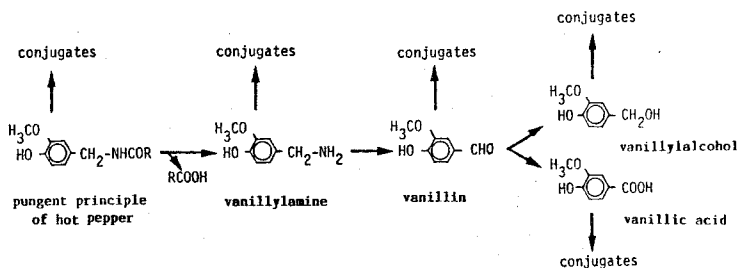
Fig.4.Proposed pathway for the biotransformation of pungent principle of hot pepper

microsomal mixed-function oxidase by enhancing the biosynthesis of

components of this system, particularly cytochrome P-450(9).

Therefore, it might be considered that the difference of enzyme

preparation led to the apparent discrepancy between my results and

those of Lee and Kumar.

Judging from the results obtained in the present experiments,

the major metabolic pathway of the pungent principle of hot pepper

in rats should be as shown in Fig.4. The pungent principle was

hydrolyzed to vanillylamine and branched-chain fatty acids. The

former compound was further received an oxidative deamination, and

then follwed to oxidation or reduction. The metabolites were

mainly excreted into the urine as glucuronide conjugates. This

excretion was almost completed within 2 days in rats. The follow-

ing metabolic pathway of vanillin was confirmed to be similar to

that of vanillin orally administered(11).

Vanillin, a metabolite of DC, is used as food additive (mini-

mum lethal dose orally in rats, 3.0 g/kg)(12). Vanillic acid and vanillylalcohol, major metabolites of DC, are metabolites of vanillin(11). Therefore, it is considered that vanillin, vanillic acid and vanillylalcohol are not toxic compounds. However, it is not known whether $Iso-C_{10:0}$ and vanillylamine are more toxic or not than the parent compound.

The present study demonstrated that DC-hydrolyzing enzyme distributed in various organs of rats, particularly in gastrointestinal tract and liver, and that DC and its metabolites were conjugated with gluculonate probably in liver. These biotransformations may be largely contributed to lower the toxicity of the pungent principle administered orally.

REFERENCES

1. S.S.Lee and S.Kumar, In Microsomes, Drug Oxidations, and Chemical Carcinogenesis, vol.2 (J.Coon et al., eds) Academic Press, New York, 1980, pp.1009-1012.

2. K.Iwai, K.R.Lee, M.Kobashi, T.Suzuki, and S.Oka, Agric. Biol. Chem., 42, 201(1978).

3. O.H.Lowry, N.Rosebrough, L.A.Ferr, and R.J.Randall, J. Biol. Chem., 193, 265(1951).

4. P.H.Williams, Int. J. Appl. Radiat. Isotopes, 19, 377 (1968).

5. K.R.Lee, T.Suzuki, M.Kobashi, K.Hasegawa, and K.Iwai, J. Chromatogr., **123**, 119(1976).

6. J.P.Thenot, E.C.Horning, M.Stafford, and M.G.Horning, Anall. Lett., 5, 217(1972).

7. J.I.Nagy, In Handbook of Psychopharmacology, vol.15, (Iverson et al. eds.) Plenum Publishing Co., New York, 1982, pp. 185-235.

8. T.Glinsukon, V.Stitmunnaithum, C.Toskulkao, T.Burannawuti, and V.Tangkrisanavinont, Toxicon., **18**, 215(1980).

9. A.H.Conney, Pharmacol. Rev., **19**, 317(1967).

10. R.G.Thurman, L.A.Reinke, and F.C.Kauffman, In Reviews in Biochemical Toxicology, vol.1, (E.Hodgson et al. eds.) Elsevier North Holland, Inc., New York, 1979, pp.249-285.

11. L.P.Strad and R.P.Scheline, Xenobiotica, 5, 49(1975).

12. M.Windholz, S.Budavari, L.Y.Stroumtsos, and M.N.Fertig, In The Merck Index, Ninth edition, Merck & Co., INC., New Jersey, 1979, pp. 9566.

CHAPTER 4

EFFECT OF PUNGENT PRINCIPLE OF HOT PEPPER ON LIPID

METABOLISM IN RATS FED HIGH FAT DIET

In the previous chapter, the author described that the pungent principle of hot pepper is readily transported through the gastrointestinal tract by nonactive transport into the portal vein and mostly excreted into urine as metabolites in rats.

Recently, the effects of dietary components on lipid metabolism have received much attention. Vegetable proteins, dietary fiber and plant xenobiotics appeared to lower the blood cholesterol(1-3). Capsaicin(CAP) is also known to have medicinal properties. Recent studies have shown that CAP exerts a lipotropic effect similar to choline in rats(4,5) and decreases total serum myocardial and aortic cholesterol levels in turkeys(6). The information about the effects of pungent principle of hot pepper on lipid metabolism is limited. The mechanism of the effect of the pungent principle on lipid metabolism is not clear.

In the present chapter, in order to examine whether the pungent principle has the effects on lipid metabolism, the author investigates by using rats fed a high fat diet containing lard.

EXPERIMENTAL

Animals and diets

TABLE 1

COMPOSITION OF EXPERIMENTAL DIETS[1]

Ingredients	High fat diet	High fat + capsaicin diet
	%	%
Casein	10	10
Starch	40	39.986
Sucrose	10	10
Lard	30	30
Soybean oil	5	5
Mineral mixture	2	2
Vitamin mixture	1	1
Cellulose	2	2
Capsaicin	-	0.014

[1] Control diet used was a commercial one (Oriental
MF diet, Oriental Yeast Co.)

Male Wistar rats(175–185 g body weight, Shizuoka Agriculcural Cooperative Assoc. for Laboratory Animals, Hamamatsu) were individually housed in stainless-steel cages with a wire-bottom in a room maintained at 22-24°C with about 50% relative humidity. The room was lighted from 06:00 to 18:00 hours.

The composition of experimental diets is given in Table 1. CAP, which purity was determined as over 99% by HPLC(chapter 1) was purchased from E.Merck(Darmstadt, G.F.R.). Two experiments were conducted. In the first experiment, there were three dietary treatment: 1)control, 2) 30% Lard, 3) 30% Lard plus 0.014% CAP. The dose of CAP used in this study was related to that usually

ingested by rural Thai people(7). The same caloric intake for the rats of three groups was maintained by adjusting the feed intake(about 15.4 g per rat in the control, about 10.5 g per rat in the 30% Lard group and 30% Lard plus 0.014% CAP group). Rats were fed each diet for 10 days. There were 15 rats per group in each experiment. In experiment 2, there were four dietary treatment: 1) 30% Lard, 2) 30% Lard plus 0.007% CAP, 3) 30% Lard plus 0.014% CAP, 4) 30% Lard plus 0.021% CAP. The same caloric intake for the rat of four groups was maintained by adjusting the feed intake(10.5 $\pm$ 0.2 g per day in each group). Thirty rats was used in this experiments. Rats were fed each diet for 10 days. In all the experiments, rats were fed a commercial stock diet(Oriental Yeast Co., Tokyo) for 3 days to allow them to adjust to the new environment, and starved for one day. Water provided ad libitum.

Sample collection

At the end of experiments, rats were fasted for 16 hours and weakly anesthetized with pentobarbital. Blood was collected by heart puncture for serum lipids, glucose, ketone bodies and lipoprotein analysis. Immediately after blood sampling, the liver was excised, washed in chilled saline solution, blotted and cooled in crushed ice. A part of the liver was used for enzyme and the rest of the liver was kept at $-30°C$ until analysis of lipid components. Perirenal adipose tissue was excised, rinsed, blotted and weighed. A part of adipose tissue was used for enzyme assay and determina-

tion of adipose cell weight. For the determination of the intestinal absorption was made at three days of experimental 1. The fecal samples were dried, weighed and pulverized. The intestinal absorption rate of lipid was determined by the method of balance study (8).

Chemical assay

Serum triglyceride was analyzed enzymically using a commercially available kit(Triglyceride G-Test, Wako Chem. Ind. Osaka). Serum ketone bodies were also analyzed enzymically by the method of Williamson(9). Serum free fatty acid and cholesterol were measured according to the method of Itaya and Ui(10), and Person et al.(11), respectively. The analysis of serum lipoproteins were performed according to the method of Narayan et al.(12) as modified by Maruyama and Kobori(13). Liver lipids were extracted by the method of Folch et al.(14). Liver total lipids were determined gravimetrically. Liver triglyceride, free fatty acid and cholesterol were measured as descrived above. DNA of perirenal adipose tissue was determined by the method of Leyva and Kelley(15).

Enzyme assay

Approximately 4.0 g of liver was homogenized in 40 ml of 0.25 M sucrose solution and centrifuged at 600 x g for 10 min. Further, the resulting supernatant fraction was centrifuged at 28,000 x g for 30 min. The resulting supernatant and pellet fractions were

used determining enzyme activity and protein. Glucose-6-phosphate dehydrogenase(G-6-PDH) in the supernatant fraction was assayed by the method of Kornberg and Horecker(16). Beta-hydroxyacyl-CoA dehydrogenase(HADH) in the pellet fraction was assayed by the method of Osumi et al.(17). Hormone sensitive lipase(HSL) and lipoprotein lipase(LPL) activities of the perirenal adipose tissue were estimated as described by Rizack(18), and Salaman and Ribinson(19), respectively.

Statistical analysis

Student's paired t-test was used for statistical analysis of the data(20).

RESULTS

Experimental 1

Table 2 and 3 summarized the results of experimental 1. There were no difference in three groups in growth of body weight. Epididymal adipose tissue weight was increased in both high fat-fed group and high fat-fed plus CAP group. CAP supplementation slightly reduced epididymal adipose tissue weight. Perirenal adipose tissue weight was also increased in both group. The supplemantation of CAP to lard diet significantly reduced peri-renal adipose tissue weight (p<0.05). The perirenal adipose cell weight was not changed by the supplementation of CAP.

The lipid from the diets was well absorbed in each group.

TABLE 2. Effect of capsaicin in rats fed high fat diets with and without capsaicin (experimental 1)[1]

	Dietary treatments		
	Control	High Fat	High Fat plus 0.014% Capsaicin
Calorie intake (Cal/10 days)	549.0	547.2 ± 2.7	548.6 ± 4.9
Growth of body weight(g/10 days)	35.7 ± 6.3^a	18.8 ± 2.8^b	18.5 ± 6.1^b
Liver weight (g)	5.30 ± 0.50^a	5.29 ± 0.64^b	5.70 ± 0.36^b
Epididymal fat pad weight (g)	1.16 ± 0.26^a	2.22 ± 0.53^b	1.93 ± 0.36^b
Perirenal fat pad weight (g)	0.36 ± 0.24^a	2.23 ± 0.55^b	1.69 ± 0.50^c
Perirenal cell weight (mg/μg DNA)	43.6 ± 0.4	43.8 ± 0.2	44.2 ± 1.2
Fat absorption rate (%)	99.6 ± 0.1	98.4 ± 0.2	98.9 ± 0.3
Serum triglyceride (mg/dl)	26.1 ± 5.6^a	41.7 ± 16.9^b	29.3 ± 13.0^a
free fatty acid (mg/dl)	6.30 ± 1.06	6.51 ± 1.21	6.45 ± 1.78
ketone bodies (μmol/dl)	98.7 ± 12.8^a	141.5 ± 16.0^b	147.9 ± 15.4^b
cholesterol (mg/dl)	104.9 ± 10.9^a	140.3 ± 46.8^b	140.2 ± 22.0^b
glucose (mg/dl)	153.7 ± 31.0^a	103.0 ± 21.9^b	118.9 ± 25.2^b
(pre-β + β)/α lipoprotein	0.39 ± 0.06	0.40 ± 0.02	0.38 ± 0.05
Liver total lipid (mg/g liver)	56.6 ± 6.9^a	82.5 ± 13.6^b	77.7 ± 10.4^b
triglyceride (mg/g liver)	9.20 ± 1.70^a	27.8 ± 6.6^b	24.4 ± 4.4^b
free fatty acid (mg/g liver)	3.95 ± 0.70	4.35 ± 0.31	4.34 ± 0.34
cholesterol (mg/g liver)	3.73 ± 0.24^a	4.58 ± 0.28^b	4.85 ± 0.64^b

[1] Mean ± SD of 15 rats.   [2] (pre-β lipoprotein + β lipoprotein)/α lipoprotein

abc Means in the same row with different superscripts differ significantly ($p < 0.05$)

TABLE 3. Effects of capsaicin administration on various enzyme activities in rat liver and perirenal adipose tissue[1]

	Control	High Fat	High Fat plus 0.014% Capsaicin
Liver			
Glucose-6-phosphate dehydrogenase (units/g liver)	1.04 ± 0.20[a]	0.76 ± 0.33[b]	1.09 ± 0.17[a]
β-Hydroxy acyl-CoA dehydrogenase (munits/g liver)	39.7 ± 9.2[a]	53.0 ± 5.7[b]	53.5 ± 9.3[b]
Perirenal adipose tissue			
Hormone sensitive lipase (free fatty acid umol/μg DNA/hr)	3.22 ± 0.79[a]	4.92 ± 0.55[b]	4.45 ± 0.52[b]
Lipoprotein lipase (free fatty acid umol/μg DNA/hr)	23.0 ± 4.4[a]	20.5 ± 0.9[b]	25.5 ± 2.2[a]

[1] Mean ± SD of 10 rats.   [ab] Means in the same row with different superscripts differ significantly ($p < 0.05$)

The absorption rate of lipid was not affected by the supplementation of CAP. Serum triglyceride was significantly increased in rats fed the high fat diet (p<0.005). There was no difference between the control and high fat plus CAP groups in serum triglyceride. The supplementation of CAP to high fat diet again significantly reduced serum triglyceride (p<0.05). Serum free fatty acid was not altered by dietary treatments. Serum ketone bodies and cholesterol were significantly higher in rats fed the high fat and the high fat plus CAP diets compared with control (p<0.001). Serum pre-beta-lipoprotein was not changed by the difference of diets.

Liver total lipid, triglyceride and cholesterol were significantly increased by feeding high fat (p<0.05). Liver free fatty acid was not changed by dietary treatments.

Liver G-6-PDH activity was significantly lower in rats fed the high fat diet as compared with control (p<0.05). CAP significantly increased the G-6-PDH activity when it was supplemented to higt fat diet(p<0.025). The liver HADH activity was significantly higher in rats fed the high fat and high fat plus CAP diets (p<0.05). The supplementation of CAP to high fat diet did not effect on the liver HADH and the perirenal adipose HSL activities, but increased the perirenal LPL activity.

Experimantal 2

Since it has been found in experimental 1 that the supple-

mentation of CAP may influence the perirenal adipose tissue weight and triglyceride, the relationship among the supplimentation of CAP and the metabolic parameters was investigated. As shown Fig.1 there was a significant correlation ($p < 0.001$) between perirenal adipose tissue weight (% of body weight) and the intake-amount of CAP ($r = -0.738$). A similar relationship was exhibited between serum triglyceride and the intake-amount of CAP ($r = -0.444$, $p < 0.02$) (Fig.2).

DISCUSSION

The results of the present study showed that the suppleme-ntation of CAP decreased the weight of perirenal adipose tissue and concentration of serum triglyceride. The results were the same that of rats fed fructose except liver triglyceride and lopo-genic enzyme activity(21).

It was reported that the absorption of lipid in rat intestine was inhibited(22). However, in the present study and other(4) using balance stuby method, the gastrointestinal absorption of lipid was not inhibited by CAP. It was not considered that the decrease of accumulation of lipid in adipose tissue resulted from the decrease of gastrointestinal absorption of lipid in rat fed high fat plus CAP. CAP administered by the intraduodenal and intragastric dose changed morphologicaly duodenal epithelial cells(23). However, it was observed by transmission electron
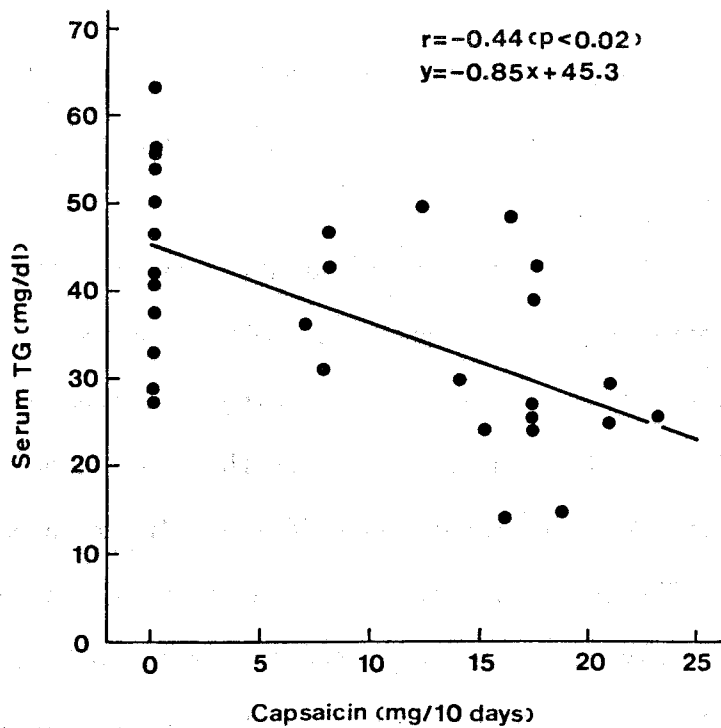
Fig.1. Relationship between capsaicin and serum triglyceride
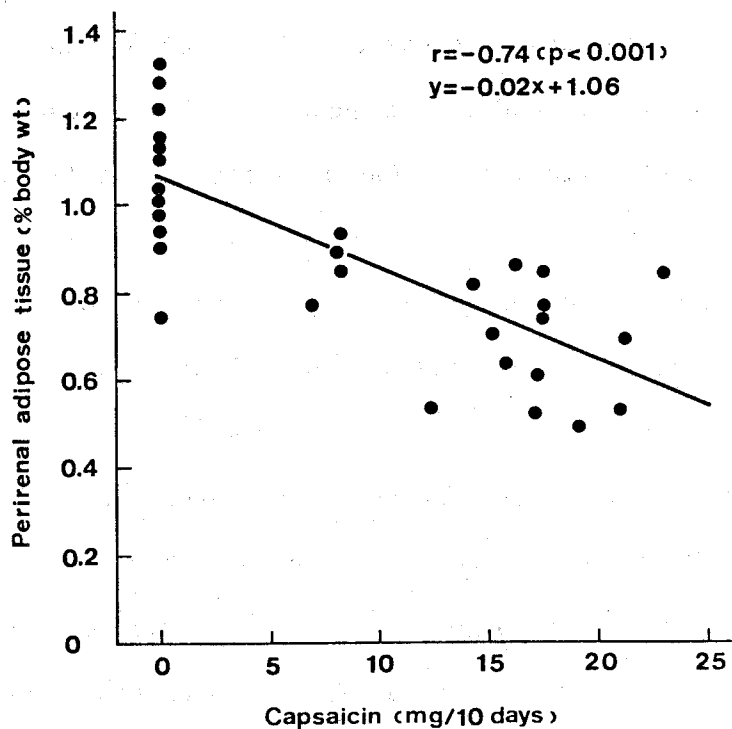
Fig.2. Relationship between capsaicin and perirenal adipose tissue

microscopic study that the supplementation of CAP(0.014%) into high fat diet did not induced the alteration of epitherial cells (unpublished observation).

Unlike the previous report using turkeys fed the cholesterol diet(6), there was no influence of CAP on the serum cholesterol concentration in the present study using rats fed no cholesterol.

Previous studies have shown that the ingestion of fat markedly inhibits hepatic lipogenesis(24,25). In my present experiments, G-6-PDH(one of lipogenic enzyme) activity in rats fed high fat diet was significantly lower than control($p < 0.05$). The enzyme activity of rats fed high fat plus CAP were the same as that of control rats. Serum triglyceride concentration of rats fed high fat plus CAP diet was decreased with a concomitant rise in the supplementation of CAP. Consequently, these results indicated that the supplementation of CAP facilitated the lipid metabolism in rats fed high fat diet. The stimulation of lipid metabolism by CAP was supported by the result of perirenal adipose tissue weight serum triglyceride level, and adipose LPL activity.

There were not difference in serum ketone bodies, pre-beta-lipoprotein, and adipose HSL activity between high fat and high fat plus CAP groups. These results suggest that the surplus energy of the nutrients taken once accumulated in adipose tissue as lipid (triglyceride). Previous studies have shown that free fatty acid was incorporated into an organ in proportion to the concentration

of fatty acid outside the organ(26,27), and the rate of fatty acid oxidation automatically increased in the organ(28,29). Thus, it is suggested that stimulation of lipid metabolism by CAP is attributable to fat mobilization from adipose tisssue. Further, this speculation was substantiated by the following experimental results using rats intraperitoneally administered CAP; increase of O_2 consumption, and up and down of respiratory quotient (see chapter 5).

It was reported that CAP influence on lipid metabolism by inhibiting lipogenic enzymes of rats fed fructose diet(21). It seems likely that a mechanism of action of CAP on lipid metabolism in rats fed fructose differs from that of rats fed lard in my present experimental condition.

References

1. K.K.Carroll and R.G.Hamilton, J. Food Sci., 40,18(1975)

2. S.Kiriyama, S.Okazaki, and A.Yoshida, J. Nutr., 97, 382 (1960).

3. A.Bordia, K.Verma, K.Vagas, and N.Bhu, Atherosclerosis, 26, 379(1977).

4. K.Sambaiah, N.Satyanayana, and L.Rao, Nutr. Rep. Int., 18, 521(1978).

5. K.Sambaiah and N.Satyanayana, J. Food Sci. Technol., 19 30(1982).

6. P.Ki, A.Negulesco, and M.Murnane, IRCS Med. Sci., 10,

   446(1982).

7. Interdepertment Committee on Nutrition for National Defense,

   Nutrition Survey-The Kingdom of Thailand. U.S. Government

   Printing Office, Washington, D.C., 1962, pp. 57-59.

8. Y.Muto, In Shoka-Kyushu, Daiich Shuppan Inc.,Tokyo, 1976,

   213.

9. H.Williamson, J.Mellanby, and A.Krebs, Biochem. J., 82,

   90(1962).

10. K.Itaya and M.Ui, J. Lipid Res., 6, 16(1965).

11. S.Person, S.Stern, and H.McGavack, Anal. Chem., 25, 813

    (1953).

12. A.Narayan, L.Creinin, and A.Kummerow, J. Lipid Res., 7,

    150(1966).

13. M.Maruyama and H.Kobori, Seibutsu-Butsuri-Kagaku, 16,

    243(1972).

14. J.Folch, M.Lees, and H.Sloame-Stanley, J. Biol. Chem.,

    226, 497(1957).

15. A.Leyva and N.Kelley, Anal. Biochem., 62, 173(1974).

16. A.Kornberg and L.Horecker, In Methods in Enzymology,

    vol.1, (S.Colowich and N.Kaplan eds.), Academic Press, New

    York, 1955, pp. 323-327.

17. T.Osumi and T.Hashimoto, J. Biochem., 83, 1361(1978).

18. A.Rizack, J. Biol. Chem., 236, 657(1961).

19. R.Salaman and S.Ribinson, <u>Biochem. J</u>., **99**, 640(1966).

20. W.Snedecor and G.Cochram, In <u>Statistical Methods</u>, 6th ed. (Japanese translated edition), Iwanani Pub. Inc.,Tokyo, 1967

21. K.Sambaiah and N.Satyanarayana, <u>J. Biosci</u>., 4,425(1982)

22. W.Nopanitaya, <u>Growth</u>, 37, 269(1973).

23. W.Nopanitaya and W.Nye, <u>Toxicol. Appl. Pharmacol</u>., **30**, 149(1974).

24. J.Dupont, <u>J. Amer. Oil Chem. Soc</u>., 42, 903(1965).

25. M.Lowenstein, In <u>Handbook of Physiology</u>, vol.7, (F.Steiner ed.), Willians & Wilkins Co., Baltimore, 1972, pp. 415-424.

26. B.Fine and H.Willians, <u>Amer. J. Physiol</u>.,**199**, 403(1960)

27. R.Evans, H.Opie, and C.Shipp, <u>ibid</u>., **205**, 766(1963).

28. B.Fritz, <u>Physiol. Rev</u>., **41**, 52(1961).

29. P.Eaton and D.Steinberg, <u>J. Lipid Res</u>., 2, 376(1961).

CHAPTER 5

ANALYSIS OF ENHANCEMENT EFFECT OF PUNGENT PRINCIPLE OF

HOT PEPPER ON LIPID METABOLISM IN RATS

As described in chapter 4, the author first found that CAP stimutated the lipid metabolism in lard-fed rats; decrease of the perirenal adipose tissue weight and serum triglyceride.

In the present chapter, the author will show the stimulating mechanism of CAP on lipid metabolism in rats by analysis of energy metabolism; O_2 consumption, respiratory quotient(R.Q.), liver glycogen, serum glucose, and serum free fatty acid.

EXPERIMENTAL

Materials

Commercial "CAP", which was pure CAP but a mixture of its analogues as determined by HPLC method(chapter 1), was purchased from E.Merck(Darmstadt, G.F.R., Lot No. 2394965). Propranolol hydrochloride (INDERAL[R] injection), pindolol (Carvisken[R] injection) and alprenolol hydrochloride (Apllobal[R] injection) were obtained from ICI Pharm. Ltd.(Osaka), Sankyo Ltd.(Tokyo), and Fujisawa Pharmaceutical Co.(Osaka), respectively. Phentolamine mesylate (Regitin[R] injection), hexamethonium bromide (Methobromin[R] injection) and epinephrin (Bosmin[R] injection) were purchased from Takeda Chemical Industries, Ltd.(Osaka), Yamanouchi

Pharmaceutical Ltd.(Tokyo), and Daiichi Seiyaku Co.(Tokyo), respectively. All other chemicals used were of guaranteed reagent grade.

Animals

Male Wiatar rats, 7-week-old, were used in experiment after feeding with purified fat diet as described in chapter 4. The rats were injected intraperitoneally CAP (3.0 mg/kg or 6.0 mg/kg), epinephrine (0.1 mg/kg) and/or blockers (propranolol; 3.0 mg/kg, pindolol; 2.0 mg/kg, alprenolol; 2.0 mg/kg, phentolamine; 10.0 mg/ kg and hexamethonium; 5.0 mg/kg). Each blocker was injected at 1 hr before injection of CAP. CAP was suspended in 0.9 wt% saline solution containing 2% ethanol and 10% Tween 80 (by volume).

Analysis of respiratory gas

A respiration apparatus used was applied the open circuit method(1). The air flow rate was 500 ml/min. Gas analysis was carried out on a measurement apparatus of respiratory metabolism, Beckman Model MAS-1 consisted of O_2 analyzer (Beckman Model 755) and CO_2 analyzer (Beckman Model 864).

Collection of liver and serum

The rats injected CAP and/or propranolol were weakly anethetized with diethyl ether just before measurement time, then were excised liver. The liver was immediately subjected to glycogen analysis. Blood was collected by heat puncture. Serum obtained after centrifugation was stored at $-30°C$ before analysis of

glucose and free fatty acid.

Chemical analysis

Liver glycogen was determined by the method of Seifter and Dayton(2). Serum glucose and free fatty acid were measured by the glucose oxidase-peroxidase method of Dahlqvist(3), and the method of Itaya and Ui(4), respectively.

Statistical analysis

Student's paired t-test was used for statistical analysis of the data(5).

RESULTS AND DISCUSSION

Increase in O_2 consumption began almost immediately after rats injected CAP (6.0 mg/kg)(Fig.1). O_2 consumption reached to its maximum level within 20-30 min, then gradually decreased and reached the primary level at about 60 min. R.Q. was about 0.80 when the rats fed high fat diet. R.Q. also began almost immediately after rats injected CAP(Fig.1). R.Q. reached to its maximum level (0.86-0.92) within 20-30 min, then gradually decreased and reached the primary level (about 0.80) at 60 min. Thereafter this constant level was maintained at least for 60 min, then R.Q. gradually decreased and reached to its minimum level at about 150 min after injection of CAP. After that R.Q. return to its primary level at about 180 min. These alterations of O_2 consumption and R.Q. by CAP were changed with the amount of injection.
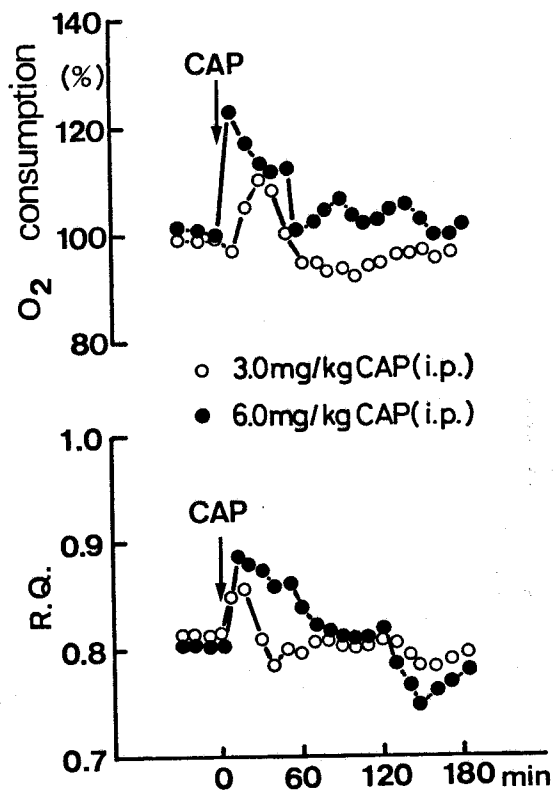
Fig.1. Effects of capsaicin on O_2 consumption and R.Q.

Figure 2 shows the level of liver glycogen, serum glucose and serum free fatty acid after dose of CAP. Liver glycogen was gradually decreased and maintained a constant level after 1 hr. Increase in serum glucose began almost immediately after rats injected CAP. Serum glucose reached to its maximum level within 2 hr, then rapidly decreased. Serum free fatty acid was gradually increased within 3 hr. The variations of these metabolic parameters well corresponded to that of image of energy metabolism
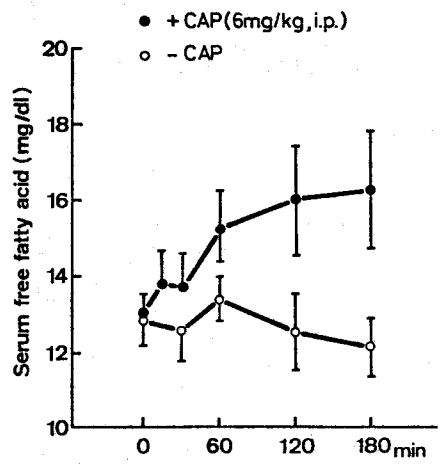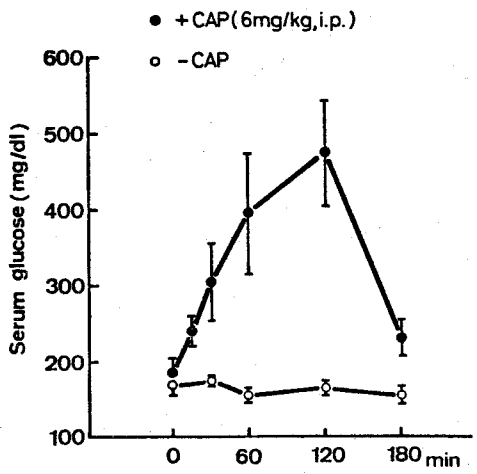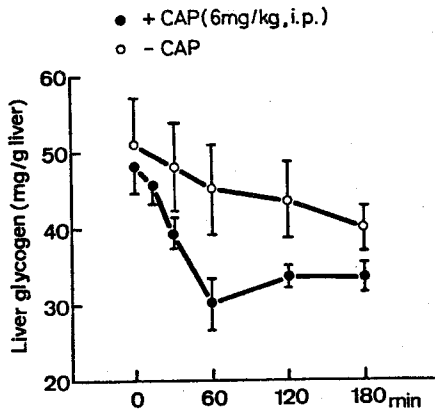
Fig.2. Effects on capsaicin on liver glycogen,
serum glucose, and serum free fatty acid.
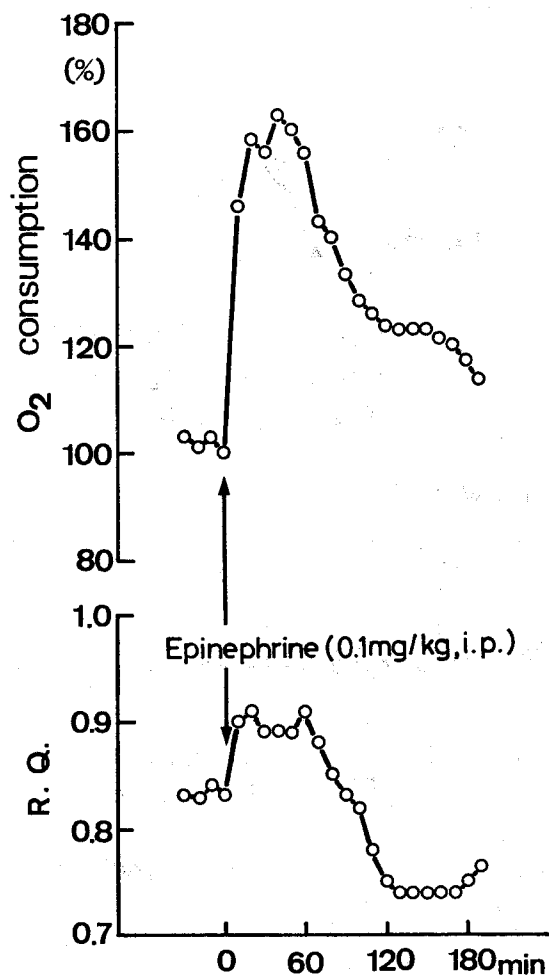
Fig.3.Effects of epinephrine on O_2 consumption and R.Q.

(CO_2 consumption and R.Q.).

Tanaka(1) recently reported that epinephrine stimulated O_2 consumption and R.Q. as a result of study by using various hormones. The variation of image of energy metabolism by dose of epinephrine was shown in Fig 3. The image of energy metabolism by
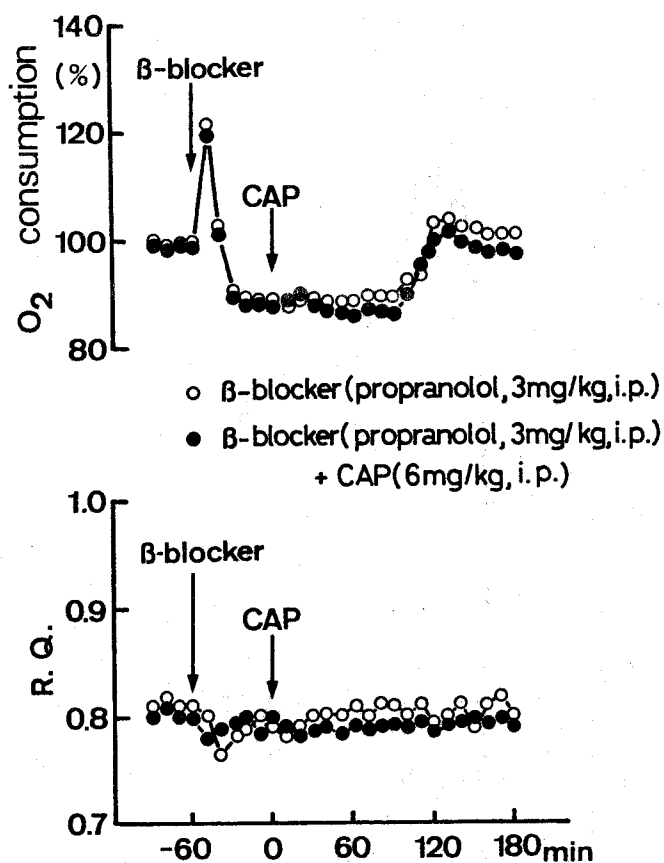
Fig.4.Effects of β-adrenergic blocker and capsaicin
on O_2 consumption and R.Q.

dose of CAP was very similar to that of epinephrine. Consequently,

to reveale a point of action of CAP on energy metabolism, the ex-

periments using various blockers were performed. The rats previ-

ously injected beta-adrenergic blocker (propranolol) were not

changed in O_2 consumption and R.Q. althrough they were injected

CAP(6.0 mg/kg)(Fig.4). Dose of other beta-blockers(pindolol and

alprenolol) also completely depressed the variation of energy

TABLE 1. Effects of β-adrenergic blocker and capsaicin on serum glucose and serum free fatty acid in rats[1]

	Control	β-Blocker[2]	Capsaicin[3]	β-Blocker + Capsaicin[4]
Serum glucose (mg/dl)	167 ± 6^a	160 ± 5^a	475 ± 70^b	180 ± 25^a
Serum free fatty acid (mg/dl)	12.4 ± 1.2^a	12.9 ± 1.0^a	16.0 ± 1.4^b	13.2 ± 1.0^a

[1] Mean $\pm$ S.D. of 4 rats   [ab] Means in the same row with different superscripts differ significantly ($p < 0.02$)   [2] The determination of parameters was done at 2 hr after injection of capsaicin(6.0 mg/kg, i.p.) into rats.
[3] The determination of parameters was done at 3 hr after injection of β-blocker (propranolol, 3.0 mg/kg, i.p.) into rats.   [4] The determination of parameters was done at 2 hr after injection of capsaicin(6.0 mg/kg, i.p.) into rats pre-injected β-blocker(propranolol, 3.0 mg/kg, i.p.) before 1 hr.

metabolism by the dose of CAP (dose not shown). The variations of metabolic parameters (serum glucose and serum free fattyacid) sig-nificuntly depressed by previous dose of beta-blocker(propranolol) (Table 1). Such variation-patters of metabolic parameters are well-known examples of epinephrine(6-8). On the other hand, previous treatment of rats by alpha-adrenergic blocker (phento-lamine) or ganglion blocker (hexamethonium) did not inhibit the variation of energy metabolism by the dose of CAP.

From the results of this experiments, it was suggested that CAP enhanced energy metabolism of rats, and its responses were based on a direct and/or indirect (via catecholamine) adrenergic actions. In chapter 4, the author described that CAP stimulated

the lipid metabolism; decrease of the perirenal adipose tissue weight and serum triglyceride in lard-fed rats. Therefore, it was speculated that the adrenergic action of CAP enhanced the lipid metabolism in rats thus this effect resulted in the decrease in the perirenal adipose tissue weight and serum triglyceride level in lard-fed rats.

REFERENCES

1. T.Tanaka, <u>J. Japanese Society of Nutr. and Food Science</u>, **31**, 1(1978).

2. S.Seifter and S.Dayton, <u>Fed. Proceed.</u>, 8, 249(1949).

3. A.Dahlqvist, <u>Anal. Biochem.</u>, 7, 18(1964).

4. K.Itaya and M.Ui, <u>J. Lipid Res.</u>, 6, 16(1965).

5. W.Snedecor and G.Cochram, In <u>Statistical Methods</u> 6th ed., (Japanese translated edition), Iwanami Pub. Inc.,Tokyo, 1967.

6. P.Ahlquist, <u>Amer. J. Physiol.</u>, **153**, 586(1948).

7. Y.Kawai and J.Arinze, <u>J. Biol. Chem.</u>, **258**, 4364(1983).

8. N.Fain and A.Garcia-Sainz, <u>J. Lipid Res.</u>, **24**, 945(1983).

SUMMARY

Chapter 1;

The qualitative and quantitative microanalyses of the pungent principle of hot pepper by HPTLC and HPLC were examined. Five analogues were well separated by HPTLC. Linearity was found at least from 20 to 200 ng. Furthermore, by using HPLC method, CAP and DC (the main components of pungent principle of hot pepper) can be determined simply and rapidly at levels from micrograms to nanograms without interferences from other things.

Chapter 2;

Gastrointestinal absorption of CAP and DC was studied in rats in vivo and in situ. Rapid absorption of CAP or DC was found from stomach and small intestine in vivo. About 85% of the dose was absorbed in the gastrointestinal tract within 3 hr. In situ, within 60 min after the administration of CAP and DC into stomach, jejunum and ileum, about 50, 80 and 70% of the respective dose had disappeared from the lumen. By using radioisotopic techniques and metabolic inhibitors, it was found that the pungent principle of hot pepper was readily transported to the portal vein through the gastrointestinal tract by a nonactive process, and partly metabolized during absorption, and that the radioactive DC and its metabolite (Iso-$C_{10:0}$) were associated with the albumin fraction in the portal blood.

Chapter 3;

The metabolism of DC in rats was investigated in vivo and in vitro using TLC, HPLC and combined GC-MS within 48 hr of oral dose of DC (6.0 mg) to male adult rats, unchanged DC and eight of its metabolites were identified in urine; i.e.,DC (8.7% of total dose) vanillylamine (4.7%), vanillin (4.6%), vanillylalcohol (37.6%) and vanillic acid (19.2%) as free and/or their glucuronide. Part of unchanged DC (ca. 10% of total dose) was excreted into feces within 48 hr. Cell-free extracts of rat liver catalyzed the hydrolysis of DC to vanillylamine and Iso-$C_{10:0}$ free fatty acid. DC-hydrolyzing enzyme activity was found in various organs of rats The metabolic pathway of the pungent principle of hot pepper in rats is proposed.

Chapter 4;

Effects of the pungent principle of hot pepper were studied in experiments using male rats fed a high fat (lard) diet. When CAP was supplemented at 0.014% of the diet, serum triglyceride and perirenal adipose tissue weight were significantlly decreased. Dietary lard decreased hepatic glucose-6-phosphate dehydroganase and adipose lipoprotein lipase activities, while the CAP supplementation increased these enzyme activity. Lipid absorption was not changed by the supplementation of CAP in a gastrointestinal

tract. The perirenal adipose tissue weight and serum triglyceride were decreased with a concomitant rise in the supplementation of CAP. These results suggest that the pungent principle of hot pepper stimulates the lipid mobilization from adipose tissue, and decreases the perirenal adipose tissue weight and serum triglyceride in lard-fed rats.

Chapter 5;

Energy metabolism in the rats intraperitoneally injected CAP was investigated. Increase in O_2 consumption was obserbed in rats injected CAP. R.Q., liver glycogen, surum glucose, and serum free fatty acid were also obserbed its alterations within 3 hr. These alterations of energy metabolism by CAP were completely inhibited by various beta-adrenergic brockers. On the other hand, the alterations were not effected by alpha-adrenergic or ganglion blocker. From the results it is strongly suggested that the pungent principle of hot pepper enhances energy metabolism of rats and its responses are based on a direct and/or indirect (via catecholamine) adrenergic actions. Therefore, it is speculated that the adrenergic action of the pungent principle of hot pepper results in the decrease of the perirenal adipose tissue weight and serum triglyceride level in lard-fed rats.

analysis.