available space, with the spermatheca becoming more densely packed with a longer time since insemination.

The most parsimonius explanation for the reported fluctuations of phenotypic frequencies, lies in the process of sperm migration and the filling of the spermatheca. Queens mate with many drones in rapid succession and receive the semen of these drones into the distensible lateral oviducts[6]. Spermatozoa migrate into the spermatheca by active and passive mechanisms over a period of about 24 h[7-9]. It is likely that spermatozoa deposited at the anterior ends of the lateral oviducts (the greatest distance from the spermathecal duct) are last to reach the spermatheca. As the spermatheca becomes more densely packed, there is less room for spermatozoa to distribute themselves and, therefore, a higher probability that like ('brother') spermatozoa will be distributed in close proximity to each other. As they become more densely packed, they may tend to become oriented parallel to each other through random movement in a confined space, thus forming the characteristic 'whorls' of sperm shown by others[10,11].

It is unlikely that instrumental insemination causes anomalous results. Taber[1] showed similar phenotypic fluctuations in both naturally mated and instrumentally inseminated queens. Furthermore, both Bishop[12] and Laidlaw[6] have shown that queens returning from mating flights have very few (if any) spermatozoa in their spermathecae, demonstrating that the male ejaculation itself does not directly fill the spermatheca.

The queen may be actively mixing some of the spermatozoa in her oviducts by contracting the abdomen and the thin muscular layer that covers the oviducts[6]. The adaptive significance of mixing these sperms would be to increase the effective number of matings[13].

Decreasing fluctuations in observed phenotypes can be explained by the sperm utilization model of Harbo[14]. Harbo presented data that suggest honeybee queens release a constant volume of spermathecal fluid each time they fertilize an egg. The spermathecal fluid is then replaced. This leads to a rapid decrease in the density of spermatozoa in the spermatheca and, hence, more potential for mixing. A rapid decrease in fluctuations is expected and is demonstrated by the data of Kerr et al.[4].

1 Taber, S., J. Econ. Entomol. *48* (1955) 522.
2 Crozier, R.H., and Brückner, D., Am. Nat. *117* (1981) 561.
3 Page, R.E., and Metcalf, R.A., Am. Nat. *119* (1982) 263.
4 Kerr, W.E., Martinho, M.R., Goncalves, L.S., Rev. Bras. Genet. *3* (1980) 339.
5 Laidlaw, H.H., Instrumental Insemination of Honey Bee Queens. Dadant and Sons, Inc., Hamilton, Ill. 1977.
6 Laidlaw, H.H., J. Morph. *74* (1944) 429.
7 Ruttner, F., Insectes soc. *3* (1956) 351.
8 Ruttner, F., and Koeniger, G., Z. vergl. Physiol. *72* (1971) 411.
9 Gesner, B., and Ruttner, F., Apidologie *8* (1977) 1.
10 Lensky, Y., and Schindler, H., Annls Abeille *10* (1967) 5; plate 4, fig. 4.
11 Ruttner, F., Enbergs, H., and Kriesten, K., Apidologie *2* (1971) 67; fig. 1a.
12 Bishop, G.H., J. exp. Zool. *31* (1920) 267.
13 Page, R.E., Genetics *96* (1980) 263.
14 Harbo, J.R., J. apic Res. *18* (1979) 204.

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Quercetin interacts with calmodulin, a calcium regulatory protein[1]

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Summary. Quercetin was found to interact with the Ca^{2+}-calmodulin complex, suggesting that it may act as a calmodulin antagonist in vivo to inhibit calmodulin-dependent phenomena caused by biologically active agents, including tumor promoters.

It has been reported that quercetin, a flavonoid, has mutagenic activity[2-6], although it was found to be non-carcinogenic in animal tests[7-9]. We have recently found that quercetin shows inhibitory effects on a wide variety of tumor promoter-induced phenomena, and suggested that the anti-tumor-promoter activity of quercetin may result in lack of development of tumors, despite its mutagenicity. Calmodulin is known to be the major calcium-regulatory protein in various kinds of cells, and is reported to participate in the mechanism of action of tumor promoters[12-16]. Therefore, it appeared interesting to investigate the effect of quercetin on the Ca^{2+}-calmodulin system to know why quercetin shows anti-tumor-promoter activity. In this communication, we present evidence suggesting that quercetin acts as a calmodulin antagonist; this characteristic may be involved in the mechanism of the anti-tumor-promoter action of quercetin.

It has been reported that fluorescence of N-phenyl-1-naphthylamine (NPN) is enhanced in the presence of Ca^{2+}-calmodulin complex, and that calmodulin antagonists depress the enhanced fluorescence[17,18]. In order to demonstrate the interaction of quercetin with calmodulin, we examined the effect of quercetin on the enhanced fluorescence of NPN induced by binding with calmodulin. Calmodulin was obtained from Boehringer Mannheim Yamanouchi Co., Tokyo. N-Phenyl-1-naphthylamine was purchased from Wako Pure Chemical Industries, Ltd., Osaka. Fluorescence measurements were carried out at room temperature using a Shimadzu fluorescence spectrophotometer (Model RF-502). As shown in figure 1, enhanced fluorescence of NPN induced by the addition of calmodulin was completely depressed by quercetin at the concentration of 50 μM, and it was suggested that quercetin interacted with calmodulin.

In order to confirm this possibility, we tried to show the direct interaction of quercetin with calmodulin; that is, the binding activity of ^{14}C-quercetin to Ca^{2+}-calmodulin complex was examined by the equilibrium binding technique of

Hummel and Dryer[19]. Figure 2A shows the elution profile for the binding experiment for calmodulin. The appearance of a ^{14}C-quercetin peak and trough in the profile, and the coincidence between radioactive peak and protein peak, indicate the binding of ^{14}C-quercetin to calmodulin.

It has been found that the binding of quercetin to proteins can occur in a non-specific manner. For example, we and other investigators observed that quercetin binds non-specifically to plasma proteins. On the other hand, specific interactions of quercetin with various enzymes were also demonstrated[20-23]. Therefore, it might be worthwhile to examine whether there is some specificity for calmodulin in the phenomenon of binding to quercetin. In this context, we examined the binding between quercetin and trypsin, another kind of Ca^{2+}-binding protein[24], in order to evaluate the binding activity of calmodulin. As shown in figure 2B, no obvious ^{14}C-quercetin peak and trough could be detected in the elution profile under the present experimental conditions. The result suggests that the binding activity of calmodulin to quercetin is relatively high compared to that of trypsin.

Furthermore, in a preliminary experiment, we found that the binding of calmodulin to quercetin was accomplished in a Ca^{2+}-dependent fashion, since the ^{14}C-quercetin peak was markedly depressed in the presence of EGTA, a Ca^{2+} chelator. Therefore, the binding to quercetin seemed to occur mainly with the biologically active form of calmodulin.

From the results obtained in this study, it is concluded that quercetin interacts directly with calmodulin, and suggested that quercetin may act as a calmodulin antagonist in vivo to inhibit calmodulin-dependent phenomena, including tumor promoter-induced changes in cellular functions.

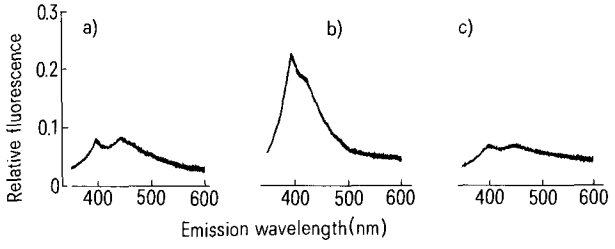

Figure 1. Effect of quercetin on the enhanced fluorescence of NPN induced by calmodulin. Fluorescence intensity was determined in 3 ml of 10 mM Tris-HCl, pH 7.5, and 0.1 mM $CaCl_2$, with *a* NPN (8 μM) alone, *b* NPN (8 μM) + calmodulin (60 μg), *c* NPN (8 μM) + calmodulin (60 μg) + quercetin (50 μM). NPN and quercetin were dissolved with ethanol, of which final concentration was 0.4%. Excitation was at 360 nm, and emission intensity was measured from 370 nm to 600 nm.

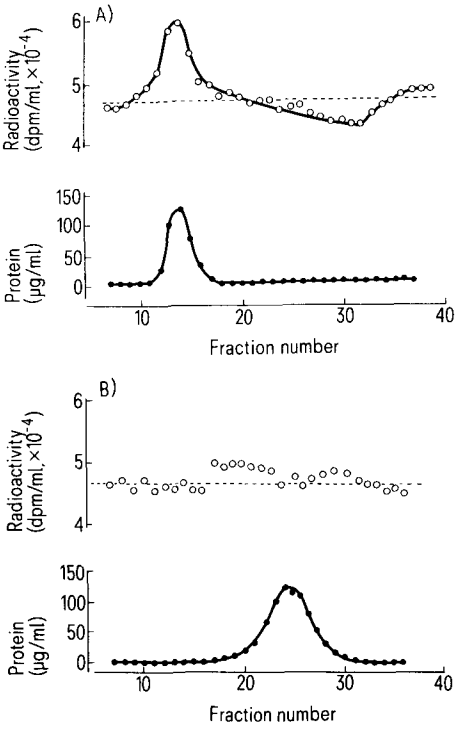

Figure 2. Elution profile for the demonstration of ^{14}C-quercetin binding to calmodulin or trypsin. Sephadex G75 (0.9 × 27 cm) was pre-equilibrated with 5 mM Tris-HCl, pH 7.5, and 1 mM $CaCl_2$ (buffer A) containing ^{14}C-quercetin (5×10^4 dpm/ml, 0.4 μg/ml, prepared by Daiichi Pure Chemicals Co., Ltd., Tokyo, purity: 98%). Calmodulin (250 μg) [A], or trypsin (250 μg) [B], dissolved in 0.5 ml buffer A was applied on the column, and gel filtration was carried out. Each fraction (0.7 ml) eluted with buffer A containing ^{14}C-quercetin was analyzed for radioactivity and protein concentration. Dotted line shows the equilibrated level of radioactivity for ^{14}C-quercetin in the column.

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2 Bjidanes, L.F., and Chang, G.W., Science *197* (1977) 557.
3 Brown, J.P., and Dietrich, P.S., Mutation Res. *66* (1979) 223.
4 Hardigree, A.A., and Epler, J.L., Mutation Res. *58* (1978) 231.
5 MacGregor, K.T., and Jurd, L., Mutation Res. *54* (1978) 297.
6 Nagao, M., Morita, N., Yahagi, T., Shimizu, M., Kuroyanagi, M., Fukuoka, M., Yoshihara, K., Matori, S., Fujino, T., and Sugimura, T., Envir. Mutagenesis *3* (1981) 401.
7 Hirono, I., Ueno, I., Hosaka, S., Takanashi, H., Matsushima, T., Sugimura, T., and Natori, S., Cancer Lett. *13* (1981) 15.
8 Morino, K., Matsukura, N., Kawachi, T., Ohgaki, H., Sugimura, T., and Hirono, I., Carcinogenesis *3* (1982) 93.
9 Saito, D., Shirai, A., Matsushima, T., Sugimura, T., and Hirono, I., Teratog. Carcinog. Mutagen. *1* (1980) 213.
10 Nishino, H., and Iwashima, A., Proc. Jap. Cancer Assoc. 41th Annual Meeting, 1982, p.71.
11 Umezawa, K., and Matsushima, T., Proc. Jap. Cancer Assoc. 41th Annual Meeting, 1982, p.71.
12 Murry, A.W., Jones, M.J., Guy, G.R., and Froscio, M., Biochem. int. *3* (1981) 1.
13 Froscio, M., Guy, G.R., and Murry, A.W., Biochem. biophys. Res. Commun. *98* (1981) 829.
14 Kwong, C.H., and Mueller, G.C., Cancer Res. *42* (1982) 2115.
15 Jones, A., Boynton, A.L., MacManus, J.P., and Whitfield, J.F., Exp. Cell Res. *138* (1982) 87.
16 Nishino, H., Iwashima, A., and Sugimura, T., in: Calmodulin and Intracellular Ca^{2+} Receptors, p.448. Eds S. Kakiuchi, H. Hidaka and A.R. Means. Plenum Press, New York 1982.
17 LaPorte, D.C., Wierman, B.M., and Storm, D.R., Biochemistry *19* (1980) 3814.
18 Epstein, P.M., Fiss, K., Hachisu, R., and Andrenyak, D.M., Biochem. biophys. Res. Commun. *105* (1982) 1142.
19 Hummel, J.P., and Dreyer, W.J., Biochim. biophys. Acta *63* (1962) 530.
20 Cantley, L.C., Jr., and Hammes, G.G., Biochemistry *15* (1976) 1.
21 Granziani, Y., Biochim. biophys. Acta *460* (1977) 364.
22 Shorshan, V., and MacLennan, D.H., J. biol. Chem. *256* (1981) 887.
23 Beretz, A., Anton, R., and Stoclet, J.C., Experientia *34* (1978) 1054.
24 Levine, B.A., and Williams, R.J.P., in: Calcium and Cell Function, Vol.2, p.1. Ed. W.Y. Cheung. Academic Press, New York 1982.

0014-4754/84/020184-02$1.50 + 0.20/0