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FLAVONOID IMPAIRMENT OF NEUTROPHIL RESPONSE*

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Abstract—Flavonoids are a class of phenolic plant pigments which impair the oxidative burst of neutrophils to an extent dependent on their hydrophobicity. The distribution of quercetin and of morin in nitrogen-cavitated neutrophils paralleled their respective hydrophobic characteristics and respiratory burst inhibition. While both flavonoids were localized primarily in the specific granule membrane of neutrophils, the amount of quercetin was considerably greater than that of morin. We here demonstrate inhibition of the initial stimulation response, depolarization of the membrane potential as monitored by fluorescence of the membrane probe diS-C_3-(5), and of the respiratory burst, monitored by following the destruction of diS-C_3-(5), a reaction mediated by the H_2O_2 produced in the burst. The flavonoids kaempferol, morin, quercetin, or fisetin were preincubated with human neutrophils at a concentration of 100 μM per 2×10^6 cells/ml for 2–3 min and subsequently stimulated with 1 μg/ml of the tumor promoter phorbol myristate acetate (PMA) or with 60 μg/ml of immune complex. The effect of each compound differed, i.e. depolarization was enhanced by some and inhibited by others, while H_2O_2 generation was inhibited by each, supporting our previous findings that membrane potential depolarization and the respiratory burst are dissociable events. Concentration–response experiments, performed at flavonoid concentrations between 12.5 and 500 μM to determine the IC_{50} values of these compounds for depolarization and burst activation, indicated that none of the flavonoids affected the resting potential, while all perturbed the stimulus-coupled response, the direction and extent of the perturbation depending upon the stimulus, and the function assessed. These data show that the effects of flavonoids on human neutrophils are complex and suggest several sites of action depending upon the flavonoid's subcellular distribution and pathway of stimulation.

Neutrophils are stimulated when specific receptors recognize a soluble agonist [e.g. formyl-methionyl peptides, concanavalin A, or phorbol myristate acetate (PMA)] or an insoluble one [immune complex or opsonized zymosan]. The proposed sequence of events upon ligand–receptor binding is: membrane depolarization [1, 2], internal calcium release [3] and degranulation [4, 5], which results in the fusion between the granules and the plasma membrane; the sequential relationship between these events remains unclear.

While resting neutrophils consume little oxygen, upon stimulation there is a marked increase in oxygen uptake [6, 7] as the oxidative enzyme system is assembled and functions to convert O_2 into various microbicidal products such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH\cdot$) or similar species, and in the presence of myeloperoxidase, hypochlorous acid [8]. Stimulation of neutrophils can, therefore, be monitored by observation of their O_2 uptake [9] or of their H_2O_2 production [10]. The NADPH oxidase enzyme system, which is responsible for the respiratory burst, is localized in the plasma membrane [11, 12]. It has been suggested that two of its components, a flavoprotein [13, 14] and cytochrome b_{-245} [15], found in a secondary [16, 17] or tertiary [18] granule, are translocated upon PMA stimulation to the plasma membrane [16, 17]. However, it has been reported recently that *b* cytochrome translocation does not correlate with fluoride anion [19] or formyl-methionyl-peptide [20] stimulation and that PMA stimulated granule-free cytoplasts exhibit the burst [21]. The exact nature and mechanism of activation of the oxidase thus remain undefined.

Phorbol myristate acetate (PMA), also known as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), is the irritant and tumor-promoting agent of croton oil, derived from the seed oil of *Euphorbiacea Croton tiglium* [22]. This phorbol ester has been shown to stimulate neutrophils by binding specifically to a receptor [23, 24] whose K_d is in the nanomolar range. The rate and extent of receptor occupancy are proportional to the rate of whole cell superoxide production [23] and of NADPH-oxidase activity [24], thereby mediating the activation of the cell's respiratory burst following exposure to PMA.

The effects of flavonoids, as potential anti-inflammatory agents, on this system have been investigated [25]. Flavonoids are phenolic plant pigments which can induce a wide range of biological effects, including enzyme inhibition, plant growth regulation and mutagenicity of bacterial testing strains [25–27]. We have shown previously that these substances impair the normal stimulation of the respiratory burst and inhibit the activity of the NADPH-oxidase [25] but do not impair PMA binding (A. I. Tauber and P. Blumberg, unpublished data). This inhibition exhibits specificity with regard to flavonoid structure and correlates with the hydrophobicity of the par-

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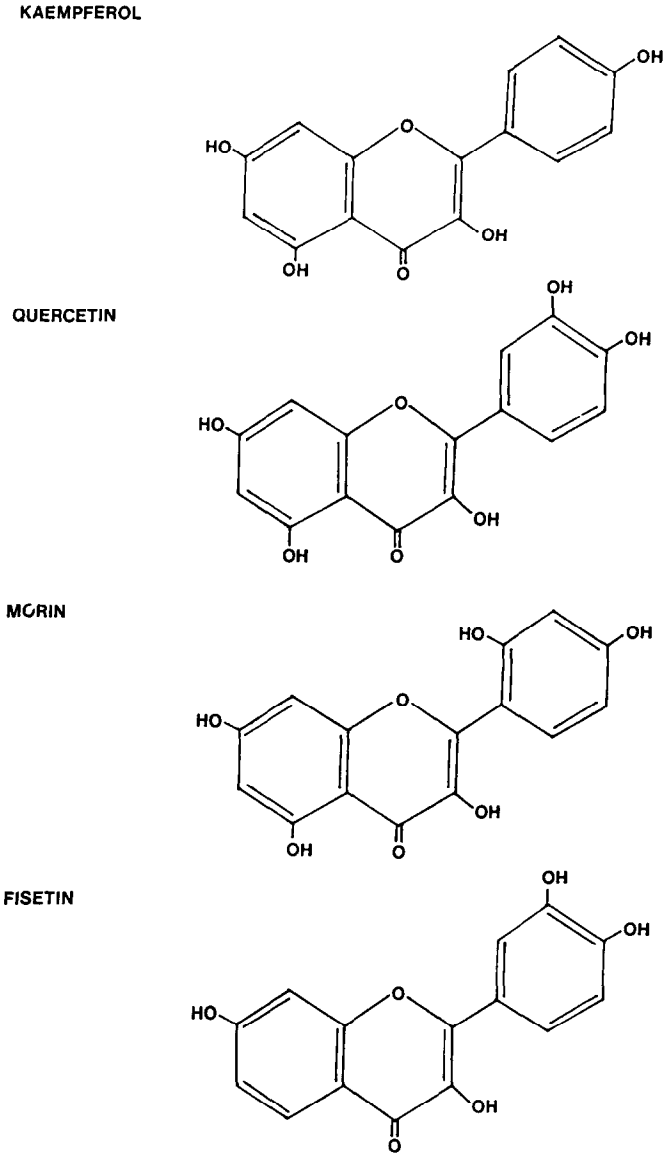


Fig. 1. Structures of the flavonoids kaempferol, quercetin, morin and fisetin.

ticular flavonoid as measured by its partition coefficient between octanol and phosphate-buffered saline [25]. The degree of inhibitory activity is also dependent on the number and/or position of hydroxyl groups on the B ring [25] (Fig. 1). Some inhibition, by quercetin, of β-glucuronidase secretion by f-met-leu-phe-stimulated rabbit neutrophils has been reported [28].

In this study we have extended our initial observations to further examine flavonoid inhibition of neutrophil function. In examining the activation pathway we found that, while the flavonoids inhibited membrane depolarization, the inhibitory profile did not strictly follow hydrophobic solubility, and there was no correlation between the membrane depolarization response and the activation of the respiratory burst. Furthermore, the preincubation with morin of PMA- or immune complex-stimulated cells appeared to shunt the molecular oxygen consumed in the respiratory burst to some as yet unidentified product. These studies suggest complex roles for flavonoids as inhibitors of neutrophil function.

MATERIALS AND METHODS

Buffers. Phosphate-buffered saline (PBS) (133 mM NaCl, 10 mM $NaH_2PO_4 + Na_2HPO_4$, 5.3 mM KCl, pH 7.4), was used with or without 4 mM glucose as indicated. Krebs–Ringer phosphate (KRP) (125 mM NaCl, 9.8 mM $NaH_2PO_4 + Na_2HPO_4$, 5 mM KCl, 1.3 mM Mg_2SO_4, 0.9 mM $CaCl_2$, pH 7.4) was used with or without 4 mM glucose.

Neutrophil function studies. Neutrophils were prepared from the blood of normal human volunteers as previously described [29]. Changes in the transmembrane potential of the neutrophils were measured as previously described [30, 31] by means

of the fluorescence of the lipophilic cation, 3,3'-dipropyl-thiodicarbocyanine iodide [diS-C_3-(5)] (for which we thank Dr. Alan Waggoner, Carnegie-Mellon University). While, in our previous papers, we utilized the net change in fluorescence at 30 sec as a measure of the neutrophil's membrane potential [32], we have found recently that the initial slope of the stimulus-induced fluorescence rise (depolarization) is a more accurate measure of stimulation [33].

The flavonoids interfere with the usual assay for superoxide production because of their non-enzymatic reduction of cytochrome *c*. However, this laboratory showed earlier that the observed decrease in diS-C_3-(5) fluorescence, after the latter's maximum value has been attained, is attributable to the destruction of the probe by the H_2O_2 generated when neutrophils are activated under aerobic conditions [33]. We show here (Fig. 2) that there was a linear correlation between the rate of superoxide production, as measured for control cells via cytochrome *c* reduction, and the rate of hydrogen peroxide formation by the same cells, as measured via the decrease in the fluorescence of the probe diS-C_3-(5) as described above. The direct chemical assay of hydrogen peroxide [34] also gave comparable results.

Preparation of immune complex (AgAb). Immune complex was prepared from lyophilized rabbit anti-bovine serum albumin antibody (anti-BSA) (Cappel Laboratories) and bovine serum albumin (Sigma Chemical Co.) in 0.1 M sodium phosphate, pH 7.0, at a ratio of 5Ab:1Ag on a weight to weight basis [35], a ratio determined as optimal by preparation of a precipitin curve. The antigen–antibody mixture was incubated for 2 hr at 37°, then overnight at 4°, centrifuged, washed three times with PBS, and kept refrigerated as a PBS suspension, pH 7.0. Protein concentrations were evaluated by the method of Lowry *et al.* [36]. Neutrophils have receptors for the F_c portion of the complexed IgG; therefore, these ratios should be calculated in terms of the IgG content. Since the molecular weights of IgG and BSA are 165,000 and 68,000, respectively, 60 μg of complex at a ratio of 5Ab:1Ag contains 55.4 μg IgG.

Flavonoids. The flavonoid compounds quercetin, morin, kaempferol and fisetin were obtained from the Sigma Chemical Co., St. Louis, MO. Stock solutions were made up in dimethyl sulfoxide (DMSO) at a concentration of 100 mM; subsequently, they were diluted 1:10 with PBS-glucose, and 10 μl of this dilution was used for each fluorescence meas-

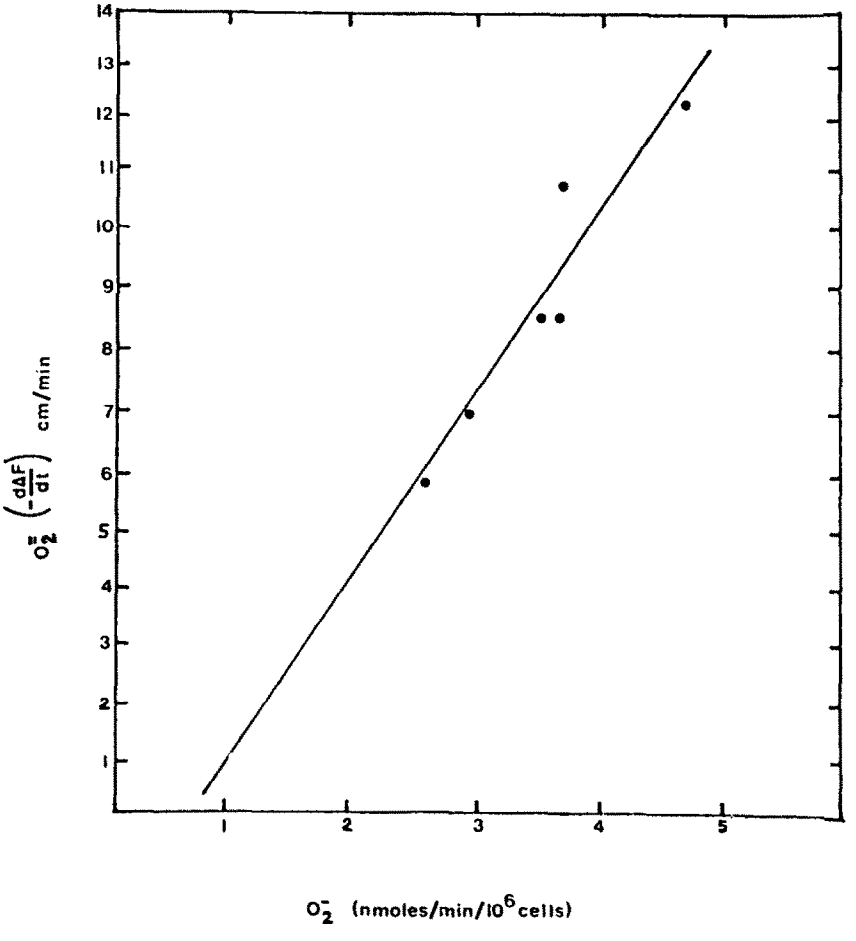


Fig. 2. Correlation between the rate of superoxide production, as measured by cytochrome *c* reduction, and the rate of hydrogen peroxide production, as measured by the destruction of the membrane potential probe diS-C_3-(5).

urement to yield a final concentration of 100 μM, except in the concentration–response experiments where the concentration range varied. The stock solutions were stored at −20° and were thawed immediately before each use.

Neutrophil organellar separation. Neutrophils were ruptured by nitrogen cavitation, and their organellar separation was achieved by Percoll density centrifugation as previously described [16]. Three distinct and well-separated bands were obtained and analyzed for flavonoid content. The samples were analyzed for flavonoid content as previously described [25] after extracting with petroleum ether, drying under N_2, and resuspending in methanol. A Waters high performance liquid chromatography system (model 272 Gradient System) was employed to elute the flavonoids from a Bondapak C-18 column (Waters) with a linear methanol/water (1% acetic acid) gradient (detection at 370 nm).

RESULTS

It has been demonstrated that one of the effects of the flavonoids is the impairment of the stimulation of the respiratory burst in human, guinea pig and rabbit neutrophils [25, 28]. Our studies with the compounds kaempferol, morin, quercetin, and fisetin have corroborated those results and have examined whether the impairment also occurs at the level of

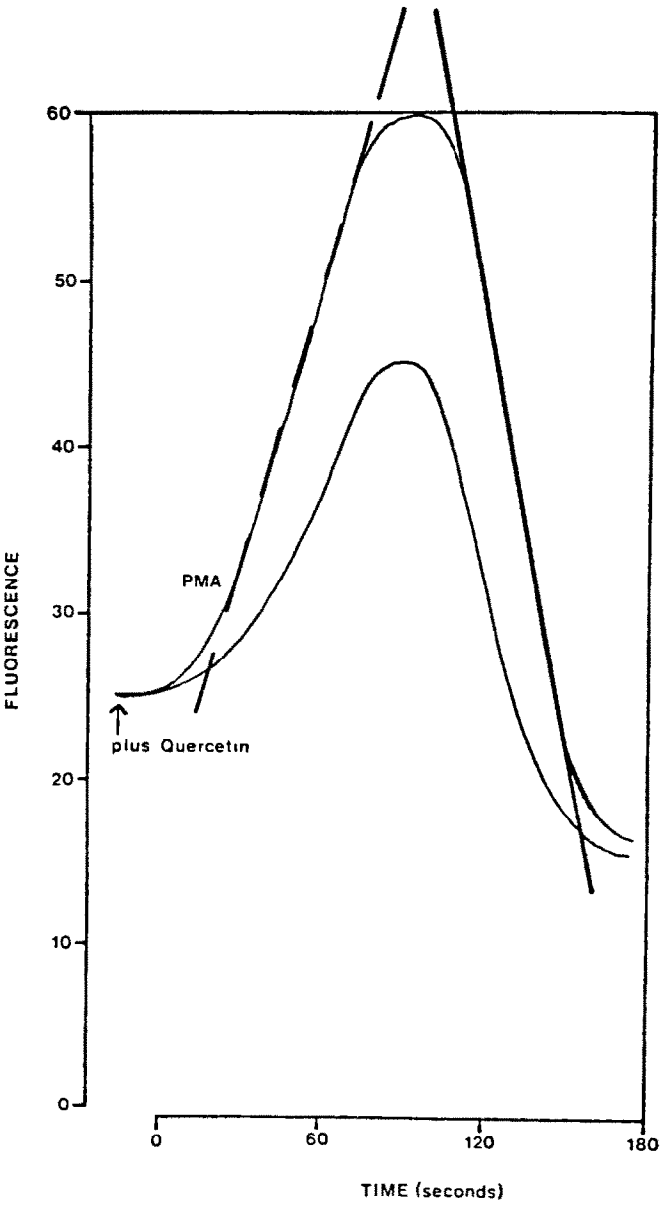


Fig. 3. Representative tracing of the fluorescence of diS-C_3-(5)-equilibrated neutrophils activated with 1 μg/ml of phorbol myristate acetate in the absence (upper curve) or presence (lower curve) of 100 μM quercetin.

Table 1. Effects of flavonoids on the neutrophil response to phorbol myristate acetate

Flavonoids*	Rate of depolarization	IC_{50} depolarization† (μM)	Rate O_2^- production	IC_{50} O_2^- production† (μM)	Hydrophobicity‡	Localization§
Kaempferol	1.59	70	0.44	12.5	5.6	
Morin	2.13	50	0.40	35	1.0	Specific granule membrane
Quercetin	1.01	60	0.34	13	15.2	Specific granule membrane
Fistin	0.03	50	0.14	18	5.6	

* The flavonoids were present at 100 μM final concentration; the stimulators were added at final concentrations of 1 μg/ml PMA.

† The IC_{50} values of depolarization and of superoxide production were evaluated as described in Materials and Methods; they are the concentrations when half-maximal inhibition occurs.

‡ Hydrophobicity was measured by the partition coefficient of the flavonoid between *n*-octanol and phosphate-buffered saline [24].

§ Localization of morin and quercetin as determined after subcellular fractionation (cf. text) [19].

stimulus-induced depolarization, a phenomenon which is observed within 5 sec of stimulus–neutrophil contact. The flavonoids were preincubated at a concentration of 100 μ per 2×10^6 neutrophils/ml without affecting the resting potential. The neutrophils were then stimulated with 1 μg/ml of PMA, or with 60 μg/ml of immune complex, and the fluorescence was monitored (Fig. 3).

Concentration–response experiments were performed at flavonoid concentrations between 12.5 and 500 μM to determine the IC_{50} values of these compounds for neutrophil membrane depolarization and hydrogen peroxide generation. Concentration–response curves were normalized with respect to the control and plotted as the relative initial rate of depolarization (v_1/v_0) versus the concentration of flavonoid. The initial rate, v_0, observed in the absence of flavonoids was used as the base value in calculating these relative rates. The IC_{50} is defined as the concentration at which one-half of the eventual maximal effect was observed. It should be noted that this was not equal to the concentration at which 50% of the control value was observed since an enhanced stimulus response was observed with some flavonoids. For example, the IC_{50} for kaempferol-treated PMNs stimulated with 1 μg/ml PMA (Fig. 4A) was 70 μM kaempferol and occurred at $v_1/v_0 = 0.6$ due to potentiation, and not at 0.5.

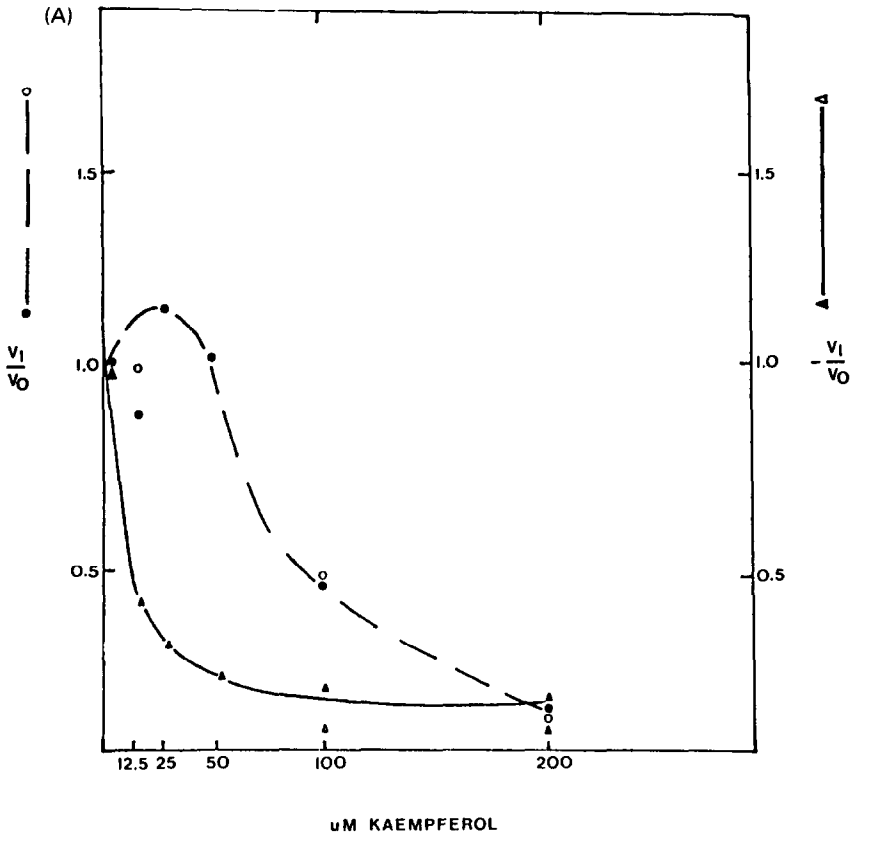
When PMA was used as the stimulus, kaempferol exhibited potentiation of both the rate and the extent of depolarization of the neutrophil membrane potential, while it led to a decrease in the rate of peroxide production. The PMA response data show that kaempferol had an IC_{50} of 70 μM for depolarization and of 12.5 μM for hydrogen peroxide formation (Fig. 4B), effects opposite to those previously noted when neutrophils were exposed to quinacrine [37]. There was no fluorescence interference by kaempferol since it exhibits a maximum emission wavelength of 440 nm with an excitation wavelength of 330 nm, while the membrane potential probe is excited at 620 nm and emits at 670 nm.

With PMA as the agonist, morin increased the rate of depolarization while slightly decreasing its extent; however, the rate of peroxide production was decreased greatly. The concentration–response curve indicates that morin exhibited considerable potentiation of membrane depolarization (i.e. increased the rate of depolarization) at the lower concentrations, with an IC_{50} of 50 μM for depolarization and 35 μM for peroxide formation (Fig. 4B). The direct chemical assay for hydrogen peroxide confirmed these findings. On stimulation with 60 μg/ml immune complex, morin inhibited the rate and the maximal extent of depolarization as well as the peroxide production (Table 1).

Quercetin decreased the rate of peroxide formation by PMA-stimulated neutrophils, while the rate of depolarization was increased. Dose–response results show that quercetin exhibited potentiation at the lower concentrations with an IC_{50} of 60 μM for depolarization and 13 μM for hydrogen peroxide formation (Fig. 4C), again confirmed by direct chemical assay of H_2O_2. On stimulation with immune complex, quercetin inhibited the rate and extent of depolarization and of hydrogen peroxide production (Table 1).

Fisetin inhibited the rate and extent of stimulation (no potentiation of PMA stimulation being detectable at any dose), and greatly decreased the rate of peroxide production. The concentration–response curve indicates that fisetin had an IC_{50} of 50 μM for depolarization and 18 μM for hydrogen peroxide formation (Fig. 4D). Fisetin almost completely abolished stimulation by immune complexes (Table 1).

Based on the hypothesis that the partition coefficients may reflect the ability of the flavonoids to penetrate into or through the neutrophil membrane, we investigated the distribution of quercetin and morin within neutrophils, using high performance liquid chromatography to determine their relative concentrations in the plasma membrane, specific granule, and azurophilic granule fractions of nitrogen cavitated neutrophils. The results of these studies (Table 1) indicate that, although both quercetin and morin were localized in the specific granules fraction, the quantities were markedly different for the two compounds.



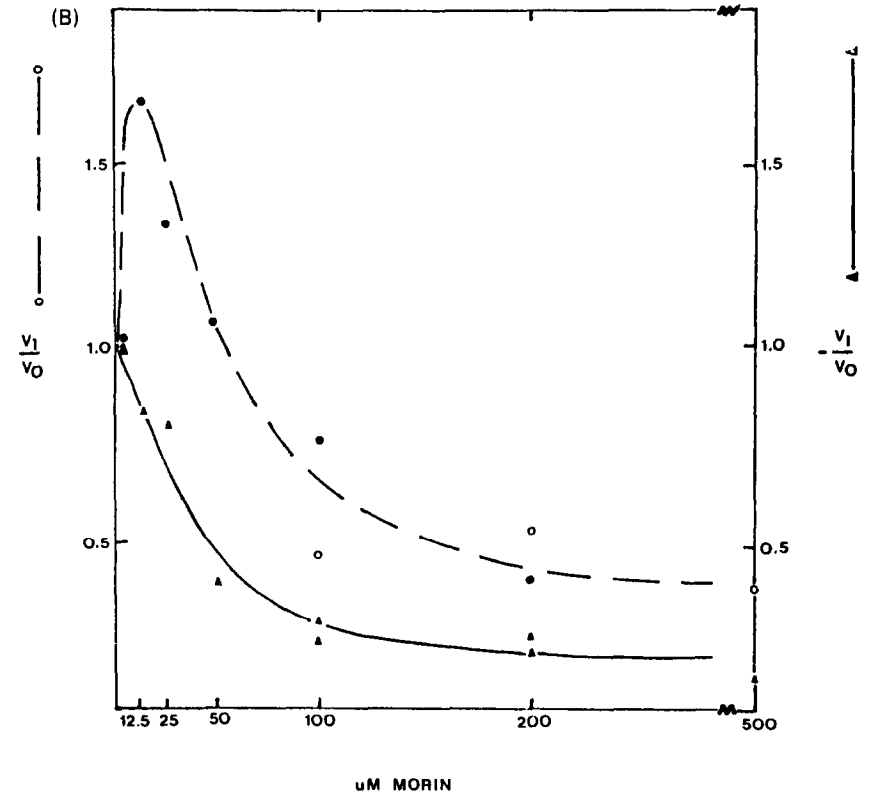


Fig. 4. (*continued*).

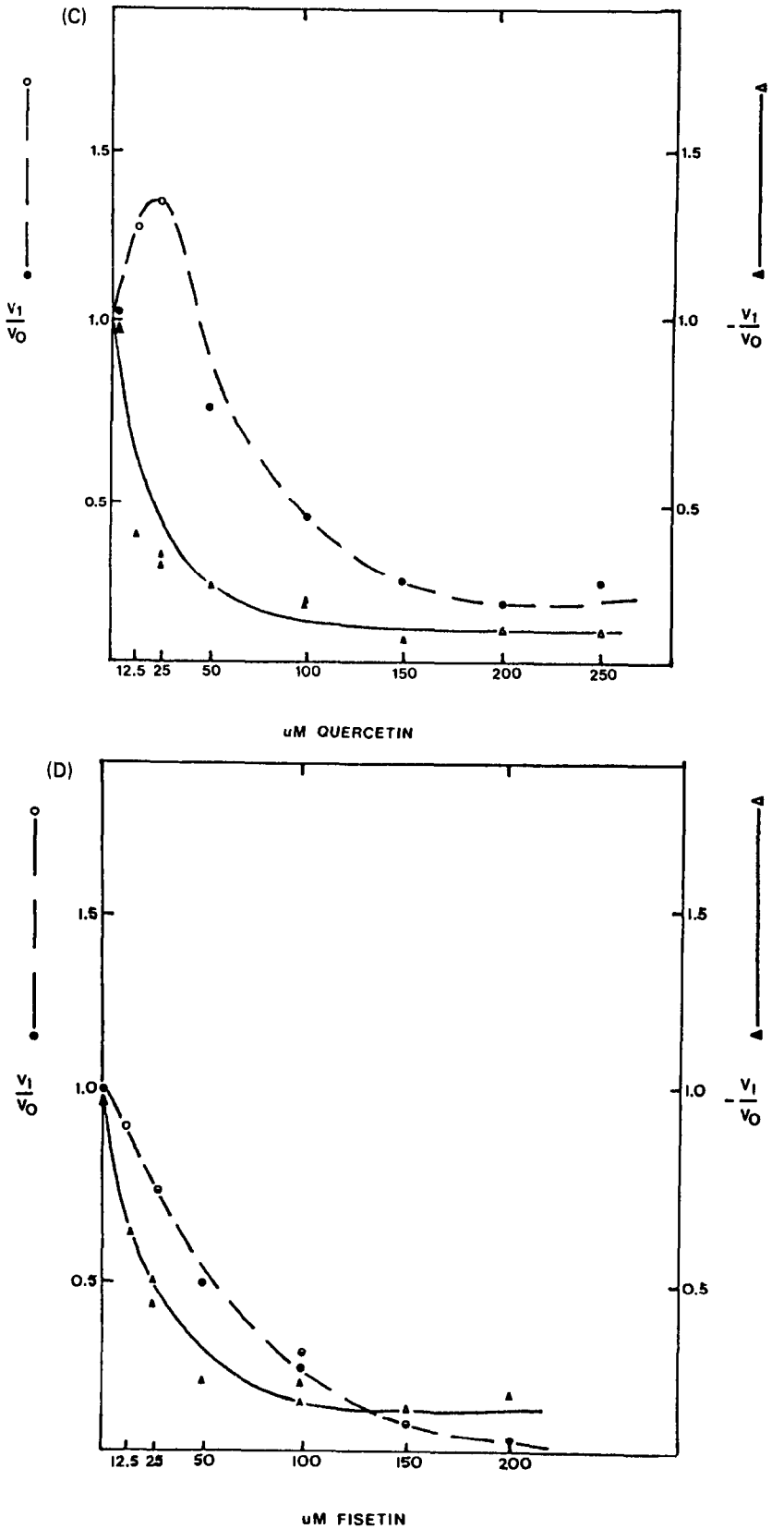


Fig. 4. Relative rate of depolarization (●——○) and of peroxide production (▲——△) in the presence of (A) kaempferol, (B) morin, (C) quercetin, or (D) fisetin. The relative rate was calculated as the ratio of v_1, the rate in the presence of flavonoid, to v_0, the rate in its absence (i.e. the control). Open and closed symbols indicate different experiments.

DISCUSSION

We have extended our preliminary studies on the perturbation by the flavonoids quercetin, kaempferol, morin and fisetin of PMA- and immune complex-stimulated human neutrophils. The anti-inflammatory effects of quercetin, kaempferol, and fisetin on histamine secretion by rat mast cells [27], as well as the numerous effects of quercetin on human neutrophils (inhibition of the ecto Mg^{2+}-ATPase on the plasma membrane [26], inhibition of the NADPH oxidase [25], of degranulation [26, 28] and of hexose transport [26]) imply that the flavonoids have a diversified capacity to interfere with the inflammatory response. The effects of the flavonoid compounds on the respiratory burst, as measured by net oxygen consumption of whole cells, can be correlated with their degrees of hydrophobicity. The hydrophobic parameter is dependent on the number and/or steric orientation of the hydroxyl groups on the B ring; inhibition, therefore, is specific with respect to flavonoid structure. Quercetin inhibited oxygen uptake, but morin did not; therefore, we postulated that these compounds may differ in their abilities to permeate the cell membrane and inhibit the NADPH-oxidase enzyme which is responsible for the oxidative burst in whole cells [25].

The NADPH-oxidase, which initiates the respiratory burst, appears to be a multi-component system; it has been suggested that two of its putative components, the cytochrome b_{-245} and associated FAD-flavoprotein, are located within the membrane of specific [15, 16] or other [18] granules. According to the applicable hypothetical mechanism, assembly is achieved when the latter becomes incorporated into the plasma membrane upon degranulation [38], thereby rendering the NADPH-oxidase capable of expressing its activity. From our results, it appears that morin and quercetin (which differ greatly in their degree of hydrophobicity) penetrate the cell membrane to a different extent although both localize in the β-fraction containing the specific granules. By intercalating into the membrane of specific granules which, upon PMA activation, fuse with the plasma membrane, the flavonoids may prevent effective activation of a functional oxidase. (While PMA-induced translocation is not inhibited under the conditions employed in these studies (A. I. Tauber and F. Higson, unpublished results), degranulation of f-met-leu-phe-stimulated rabbit neutrophils reportedly is inhibited [28].)

The flavonoids inhibit membrane depolarization as assessed by fluorescence changes of the membrane probe 3,3′-dipropyl-thiodicarbocyanine iodide. The hydrophobic nature of the flavonoids could alter the cation permeability of a membrane into which they have penetrated. As shown here, quercetin, morin and kaempferol exhibited potentiation of the depolarization of the neutrophil membrane potential at the lower concentrations (12.5 and 25 μM), i.e. they facilitated passive cation influx, which we originally thought might be attributable to their hydrophobic nature. Quercetin, kaempferol and fisetin attained maximal inhibition of the transmembrane potential at a concentration of about 200 μM, exhibiting relative initial rates of depolarization of 0.2, 0.125, and 0.02, respectively, while morin achieved maximal inhibition at 500 μM, with a relative initial slope of 0.38. Both the slope of the change in transmembrane potential and that of the change in the rate of H_2O_2 formation were normalized with respect to untreated controls; relative slopes were hence calculated. Our findings may indicate, therefore, that morin enters the membrane less effectively than the other compounds. However, fisetin, which exhibits a hydrophobicity intermediate between the other three flavonoids tested, did not demonstrate any depolarization potentiation, implying that other physicochemical factors may play a role in depolarization inhibition.

Finally, these studies further demonstrate that the respiratory burst and membrane depolarization are dissociable events as shown by the difference between the concentration–response curves for these functions. At 100 μM concentrations kaempferol, morin and quercetin tended to increase, i.e. potentiate, the rate of depolarization, and to decrease the rate of H_2O_2 production. This phenomenon confirms our previous conclusion that membrane potential depolarization and oxidative burst activity are dissociable events [37], but the mechanistic explanation is not yet understood. Further complicating the diverse functional and concentration-dependent effects of these compounds is the finding that morin-treated cells appear to shift the metabolic fate of oxygen consumed in the respiratory burst from superoxide to some other product. The nature of this shift is still under investigation. Neutrophils stimulated with unopsonized latex particles [34] or macrophages stimulated with specifically opsonized erythrocytes [39], generate H_2O_2 at the expense of superoxide. Morin may serve as a useful pharmacologic probe to further define this metabolic pathway and explain the unique stoichiometries of oxidative product formation.

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