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Studies on the Inhibitors of the Sodium-Potassium Activated Adenosinetriphosphatase. I. Effect of Phlorizin and Related Natural Products

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Investigations were performed on the correlation between the inhibitory power on Na^+, K^+-ATPase activity of phlorizin, asebotin, coreopsin, naringin- and hesperidin-chalcone and their chemical structure.

Both chalcone and dihydrochalcone inhibited Na^+, K^+-ATPase activity, depending upon the number and position of phenolic hydroxyl group in their carbon skeleton. Free hydroxyl group at 4′ position of phlorizin played an important role in the inhibitory power.

Skou[2] reported that the sodium-potassium activated adenosinetriphosphatase (Na^+, K^+-ATPase, E.C. 3.6.1.3) purified from beef brain was inhibited by sulfhydryl blocking reagents such as *p*-chloromercuribenzoic acid and the inhibition was reversed by the addition of cysteine. It is clear from these facts that sulfhydryl groups of the enzyme are involved in its activity.

Through the studies on the inhibitors of thiol-enzymes and the properties of sulfhydryl groups of enzymes, 2-methyl-1,4-naphthoquinone (K_3) (I) was found in our laboratory[3,4] to react easily with the sulfhydryl groups of the enzyme protein under mild conditions. The reactivity of K_3 with sulfhydryl groups was probably due to the presence of α,β-unsaturated ketone in its chemical structure. Ethacrynic acid (2,3-dichloro-4-(2-methylene-butyryl) phenoxy acetic acid) (II), which is diuretic and contains α,β-unsaturated ketone in the structure, was found to be inhibitory on the membrane-bound ATPase activity of kidney cortex of guinea pig.[5]

Our present investigations were performed on the effect of the chalcones, β-phenylacrylophenone (III), whose structure resembles K_3 in a sence of α,β-unsaturated ketone having electron negative groups at its both end. Robinson[6] reported that phlorizin, one of the dihydrochalcones, β-phenylpropiophenone (IV), inhibited Na^+, K^+-ATPase activity. It has been the subject of great interest that the inhibition of membrane-bound ATPase is intimately

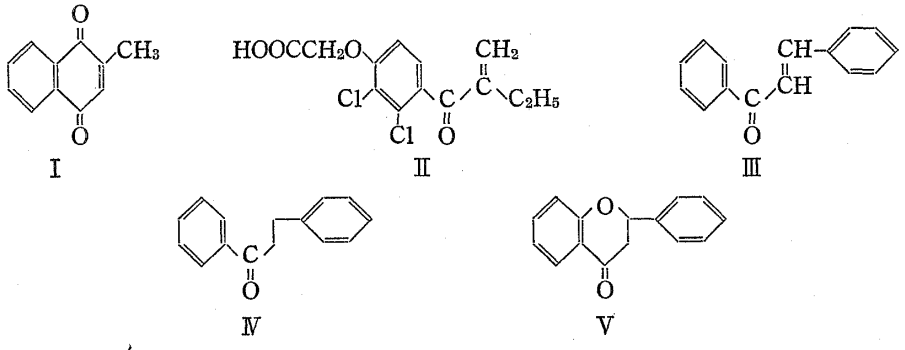


Chart 1

1) Location: *3190, Gofuku, Toyama.*
2) J.C. Skou and C. Hilberg, *Biochim. Biophys. Acta,* **110**, 369 (1965).
3) N. Nakai and J. Hase, *Chem. Pharm. Bull.* (Tokyo), **16**, 2234 (1968).
4) N. Nakai and J. Hase, *Chem. Pharm. Bull.* (Tokyo), **19**, 460 (1971).
5) D.E. Duggan and R.M. Noll, *Arch. Biochem. Biophys.*, **109**, 388 (1965).
6) J.D. Robinson, *Mol. Pharmacol.*, **5**, 584 (1969).

involved in the inhibition of transport mechanism across animal membrane.[7] In our present study, we examined the effect of derivatives of III, IV and flavanone chalcones on Na^+, K^+-ATPase activity of outer medulla of pig kidney with a view toward the correlation between the inhibitory power and the chemical structure of these compounds.

Material and Method

Na^+, K^+-ATPase Preparation——The enzyme was prepared from pig kidneys according to the procedure of Jørgensen and Skou.[8] The outer cortex and the outer medulla were dissected, placing the transverse sections of the kidney on a frozen block of 0.03M histidine, 0.25M sucrose solution, pH 7.2 (h–s). The homogenates (10 w/v % in h–s) were centrifuged at $7000 \times g$ for 15 min and the sediment was resuspended by homogenization in h–s and centrifuged again at $7000 \times g$ for 15 min. The combined supernatants were centrifuged at $48000 \times g$ for 30 min to obtain the microsomal fraction. The pellet was suspended by homogenization in h–s to a concentration of 2.5 mg protein per ml, which was divided to 2.0 ml aliquots in test-tubes and was stored at −20°. Before use, the aliquot of the microsomal fraction was incubated with 1.2 mg sodium deoxycholate per ml, 2 mM EDTA and 25 mM histidine, pH 7.0 at 20°, for 30 min. Deoxychlolate treatment was stopped by lowering the mixture solution to 0° and by diluting it with h–s. Na^+, K^+-ATPase from beef brain was prepared according to the method of Schwartz, *et al.*[9,10]

Enzyme Assay——The reaction mixture contained 3 mM $MgCl_2$, 130 mM NaCl, 20 mM KCl, 3 mM ATP (sodium salt), 30 mM histidine (pH 7.5) and the enzyme in a final volume of 2.0 ml. After 30 min at 37°, the reaction was stopped with 1 ml of 10% TCA to remove the protein by centrifugation. The inorganic phosphate in the supernatant was measured by the method of Martin–Doty.[11] The Na^+, K^+–ATPase activity was calculated as the difference in activity in the presence and in the absence of sodium and potasium ions.

Measurement of Apparent I_{50} Value——The compounds tested was dissolved in 0.1N sodium hydroxide before the measurement and diluted to the desired concentration. The apparent I_{50} value was measured from the graph of the observed inhibition percentage versus the inhibitor concentration in duplicate assays. Protein concentrations were determined by the method of Folin–Ciocalteu[12] with a minor modification, using bovine serum albumin as a standard protein.

Coreopsin, naringin, hesperidin and their chalcones were kindly provided by Dr. N. Morita and asebotin, asebogenin and their derivatives by Dr. H. Meguri. All other chemicals were of analytical reagent grade. Solutions were prepared in distilled water from an all-glass apparatus.

Result and discussion

The results obtained were summarized in Table I on the chemical structure and the inhibitory powers of phlorizin, asebotin, coreopsin, naringin- and hesperidin-chalcones, and some of their aglycones and derivatives on Na^+, K^+-ATPase activity. Apparent I_{50} value of phlorizin was measured to be 4.5×10^{-5}M and that of its aglycone, phloretin, was almost the same. This I_{50} value agreed well with that of phlorizin obtained by Robinson[6] on the Na^+, K^+-ATPase prepared from rat brain. He also reported that the phlorizin stimulated K^+-dependent *p*-nitrophenylphosphatase activity, which we will investigate in the succeeding paper.

The most striking results obtained was the marked difference of inhibitory power between phlorizin and asebotin which is 4′-methyl derivative of the former. It is clear from this finding that free hydroxyl group at 4′ position plays an important role in the inhibition of the enzyme activity. This observation was equally true of the corresponding aglycones, phloretin and asebogenin. As far as these two dihydrochalcone derivatives are concerned, aglycones were more inhibitory than their glucosides. Acetyl or methyl derivatives of asebotin and of asebogenin showed little inhibitory effect, because of their low solubility in water.

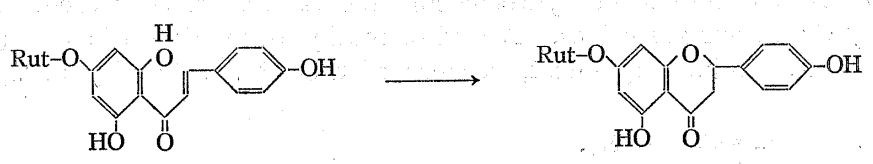
7) J.C. Skou, *Phys. Rev.*, **45**, 596 (1965).
8) P.L. Jørgensen and J.C. Skou, *Biochem. Biophys. Res. Commun.*, **37**, 39 (1969).
9) G.E. Lindenmayer and A. Schwartz, *Arch. Biochem. Biophys.*, **140**, 371 (1970).
10) H. Matsui and A. Schwartz, *Biochim. Biophys. Acta*, **128**, 380 (1966).
11) J.B. Martin and D.M. Doty, *Anal. Chem.*, **21**, 965 (1949).
12) O. Folin and V. Ciocalteu, *J. Biol. Chem.*, **73**, 627 (1927).

Table I. Chemical Structure and Inhibitory Power of Phlorizin and Related Natural Products

Materials	Formula	Chemical structure: Substituted group	Chemical structure: Skeleton	Inhibitory powder (M)
Phlorizin	$C_{21}H_{24}O_{10}$	4,4′,6′-trihydroxy-2′-O-β-D-glucosyl-	IV	apparent I_{50} 4.5×10^{-5}
Phloretin	$C_{15}H_{14}O_5$	4,2′,4′,6′-tetrahydroxy-	IV	apparent I_{50} 4.0×10^{-5}
Asebotin	$C_{22}H_{26}O_{10}$	4,6′-dihydroxy-4′-methoxy-2′-O-β-D-glucosyl-	IV	no inhibition (1.0×10^{-3})
Asebotin diacetate	$C_{26}H_{30}O_4$	4,6′-diacetoxy-4′-methoxy-2′-O-β-D-glucosyl-	IV	11% inhibition (saturated)
Asebogenin	$C_{16}H_{16}O_5$	4,2′,6′-trihydroxy-4′-methoxy-	IV	40% inhibition (1.2×10^{-4})
Asebogenin triacetate	$C_{22}H_{22}O_8$	4,2′,6′-triacetoxy-4′-methoxy-	IV	no inhibition (saturated)
Asebogenin dimethylether	$C_{18}H_{20}O_5$	2′-hydroxy-4,4′,6′-trimethoxy-	IV	15% inhibition (saturated)
Coreopsin	$C_{21}H_{22}O_{10}$	3,4,2′-trihydroxy-4′-O-β-D-glucosyl-	III	apparent I_{50} 1.7×10^{-4}
Dehydroacebegenin triacetate	$C_{22}H_{20}O_8$	4,2′,6′-triacetoxy-4′-methoxy-	III	4% inhibition (saturated)
Naringin chalcone	$C_{27}H_{32}O_{14}$	4,2′,6′-trihydroxy-4′-O-rutinosyl-	III	apparent I_{50} 2.0×10^{-3}
Hesperidin chalcone	$C_{28}H_{34}O_{15}$	3,2′,6′-trihydroxy-4-methoxy-4′-O-rutinosyl-	III	no inhibition (2×10^{-4})
Naringin	$C_{27}H_{32}O_{14}$	5,4′-dihydroxy-7-O-rutinosyl-	V	19% inhibition (1.0×10^{-3})
Hesperidin	$C_{28}H_{34}O_{15}$	5,3′-dihydroxy-4′-methoxy-7-O-rutinosyl-	V	no inhibition (8.3×10^{-4})
Ouabain				apparent I_{50} 8.8×10^{-7}
Chlorpromazine				apparent I_{50} 1.5×10^{-5}
Ethacrynic acid				appraent I_{50} 8.3×10^{-4}

Coreopsin, one of the plant chalcone pigments, showed moderate inhibitory effect. Hydroxyl group at 4′ position, which may be of importance as mentioned above, was substituted with a glucose moiety in this case. The inhibitory power of coreopsin may be attributable to the effect of the other three phenolic hydroxyl groups.

The chalcone derivatives containing 2′,6′-dihydroxyl group, in general, are chemically isomerized with ease to the corresponding flavanones, but the chalcones containing 2′ or 6′-monohydroxyl group are difficult to isomerize to the flavanones. Shimokoriyama[13] investigated the effect of pH on the isomerization reaction of naringin- and hesperidin-chalcone and reported that both chalcones converted to the corresponding flavanone, and in the case of naringin chalcone the ring-closure reaction was completed within twenty minutes at pH 7.0, and the reaction rate reduced in the more acidic medium and such an isomerization was not observed at pH 3.0. Naringin chalcone showed a weak inhibitory power and hesperidin chalcone showed no inhibitory action. A part of both chalcones may be isomerized to naringin and hesperidin because pH of the incubation mixture was 7.5. Both of these flavanones were not inhibitory. Therefore, naringin chalcone which was still remained without isomerization during the incubation period was considered to be inhibitory on the Na^+, K^+-ATPase activity.



naringinchalcone — naringin

Chart 2

13) M. Shimokoriyama, *J. Am. Chem. Soc.*, 79, 4199 (1957).

The studies on the correlation between the chemical structure of flavanone related compounds and their inhibitory power on Na^+, K^+-ATPase are now in progress.

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