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INHIBITION OF LENS ALDOSE REDUCTASE BY FLAVONOIDS—THEIR POSSIBLE ROLE IN THE PREVENTION OF DIABETIC CATARACTS

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Abstract—Flavonoids, also referred to as vitamin P, were found to be highly potent inhibitors of aldose reductase, the enzyme that initiates cataract formation in diabetes. Over forty flavone derivatives were tested and found to be active but the two most potent ones were quercitrin and quercitrin 2″-acetate, which inhibit the enzyme activity by 50 per cent at 10^{-7} and 4×10^{-8} M respectively. The potency of these two compounds surpassed that of all the previously known inhibitors of aldose reductase. Studies were conducted to determine how structural alteration in the basic flavonoid moiety affected their inhibitory activity. It is possible that further search may reveal even more potent analogues of this ubiquitously distributed group of plant polyphenols which may ultimately be useful in diabetic patients.

Flavonoids are among the most ubiquitously distributed compounds in the plant kingdom. The group consists of anthocyanins, catechins, flavones and flavanones. The primary structural feature of these heterocyclic compounds is a C_6—C_3–C_6 combination, all being derivatives of 2-phenyl chromone [1]. The various members differ from each other in the state of oxidation of the C_3 portion and the extent of hydroxylation. In many, one or more hydroxyl groups are glycosylated. A biological function of this group of compounds in man and animals was first suggested by Szent-Gyorgi *et al.* [2, 3], who reported that the flavonoids present in paprika skin and citrus peals were effective in preventing capillary bleeding and fragility associated with scurvy. This led them to designate these compounds as vitamin P. However, despite numerous clinical and experimental studies conducted during the next two decades, definite conclusions on the relationship of flavonoids with capillary permeability could not be established [4, 7] and interest in vitamin P waned.

Recently, there appears to be a resurgence of interest in flavonoids since some compounds of this group have been reported to affect membrane transport [8, 9], glycolysis [9–12], fatty acid metabolism [12] and mucopolysaccharide production [13, 14]. Our interest in flavonoids stems from studies on the mechanism of sugar cataract formation. It has been shown previously that cataract formation in diabetes and galactosemia is triggered by the accumulation in the lens of excessive sorbitol or dulcitol synthesized by the action of aldose reductase on glucose or galactose [15–17] respectively.

The formation of cataracts in galactosemic rats has been reported to be arrested by feeding them 1,2-dioxo-1H-benz-[de]-isoquinoline-2-(3H) acetic acid, an aldose reductase inhibitor referred to as AY-22,284 [18]. Since the inhibitors of aldose reductase are potentially clinically useful considerable interest has developed in seeking potent inhibitors. As shown in our preliminary report, some flavones have a striking inhibitory action on lens aldose reductase [19]. In the present paper, we present the extension of our initial study conducted in search of aldose reductase inhibitors of sufficient potency so as to be useful in the treatment of diabetic cataracts. The report covers most types of flavonoids and the biogenically related coumarins.

MATERIALS AND METHODS

Most of the compounds used in this study were obtained from commercial houses. The ones obtained from noncommercial sources are described in the tables.

Animals used in all experiments were Sprague–Dawley rats.

The preparation of aldose reductase and determination of its activity were essentially conducted as described previously [20]. The substrate used was DL-glyceraldehyde and the activity was expressed as the rate of $O.D._{340}$ nm due to utilization of NADPH in the reaction:

$$\text{glyceraldehyde} + \text{NADPH} \xrightarrow{\text{aldose reductase}} \text{glycerol} + \text{NADP}$$

The reaction mixture contained: 0·1 M phosphate buffer, pH 6.2; NADPH, 2.5×10^{-4} M; DL-glyceraldehyde, 1.5×10^{-3} M; and the enzyme. The total volume of the reaction mixture was 1 ml. The reference blank consisted of all the above compounds except the substrate. The effect of inhibitors on the enzyme activity was determined by including in the reaction mixture the compound being tested at the desired concentration. Appropriate blanks were run to correct for nonspecific reduction of NADPH and absorption by the compounds tested. The usual rate of aldose reductase activity measured was 0.052 ± 0.004 $O.D._{340}$ units/5 min of the reaction time. Most of the compounds studied were poorly water soluble. It was possible, however, to dissolve them by first suspending them in water and then

adding a 2% solution of sodium carbonate in 0.1 N sodium hydroxide in small increments. When the solution was complete, the pH was adjusted to 7.0. Usually a 10^{-3} M solution was prepared and diluted as desired.

The effectiveness of inhibitors under conditions that lead to cataract formation was studied by incubating the lens in the culture medium containing 30 mM xylose for a period of 4 hr at 37° [21]. The medium composition was as described previously [22]. Xylose was included by equivalent replacement of sodium chloride. After incubation, lenses were homogenized in 1 ml of 5% $ZnSO_4$-7 H_2O, after which an equivalent amount of 0.3 N $Ba(OH)_2$ was added. The contents were then centrifuged and an aliquot of the supernatant was lyophilized. Xylitol was determined in the residue by the gas–liquid chromatographic procedure described before [21].

Rubidium uptake was measured by incubating rat lenses in a culture medium [22] containing ^{86}Rb for a period of 16 hr by the method described in Ref. [23]. Lactate was determined enzymatically using lactate dehydrogenase and NAD [24]. The reaction was carried on in the presence of semicarbazone in order to prevent reoxidation of NADH by pyruvate. The NADH formed was measured spectrophotometrically. ATP was estimated in the boiling water extracts of lenses by the reaction with luciferin and luciferase [25] present in the firefly lantern preparation obtained from Sigma. The Aminco Chem Glow photometer was used to measure the fluorescence.

RESULTS

All the flavonoids tested were found to inhibit lens aldose reductase. The results of a survey of 44 flavonoids and their derivatives studied are summarized in Tables 1–8. The compounds are grouped in these tables primarily according to the degree of hydroxylation and the state of oxidation in ring B.

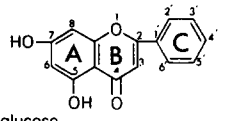
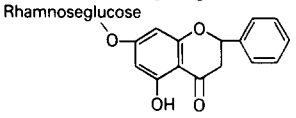
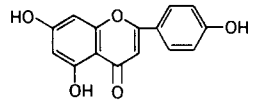
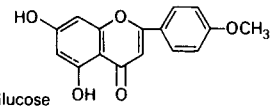
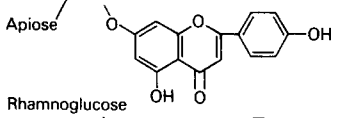
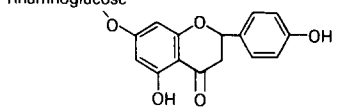
The simplest flavonoid tested was the dihydroxy flavone, chrysin (Table 1). It inhibited aldose reductase by about 50 per cent at 10^{-5} M. The addition of a third —OH group in ring C increases the inhibitory activity, as shown by results with the trihydroxy flavones. Apigenin, for example, is more potent than chrysin. The former inhibits the enzyme by 46 per cent at 10^{-6} M, whereas the latter has little effect at this level. That the hydroxyl group in ring C enhances the activity is further suggested by 4′-methoxy apigenin, which is a less potent inhibitor than apigenin itself. The phenomenon is also illustrated by naringin (7-*O*-rhamnoglucoside of 2,7,4′-trihydroxy flavanone), which is superior to that of saratonoside (7-*O*-rhamnoglucoside of 5,7-dihydroxy flavanone), in its potency as an inhibitor. Furthermore, glycosylation of the 7—OH group itself reduces the inhibitory activity as shown by apiin, the 7-*O*-glycoside, which is less active than the aglycone apigenin.

The effect of the addition of the fourth hydroxyl group on the inhibitory action of flavones was studied next (Table 2). It appears that, on the whole, tetrahydroxy flavones are no better inhibitors than the trihydroxy flavones. The inhibitory activity of the tetrahydroxy flavone luteolin is similar to that of apigenin, both inhibiting the enzyme by 45 per cent at 10^{-6} M. However, one thing obvious from the results in this table is that the catechol orientation in ring C improves the inhibitory activity. For example, fisetin, eriodictyol, orientin and luteolin all are more potent than the four kaemferol derivatives studied. The abolition of the double bond between 2,3 also leads to a loss of the inhibitory activity since 2,3-dihydroluteolin is significantly less potent than luteolin.

No conclusion about the effect of glycosylation on the inhibitory activity of flavonoids could be drawn from the study of the tetrahydroxy flavones so far used. Limited results tend to suggest that glycosylation of the third hydroxyl and C-glucosylation of the eighth carbon are without any significant effect. The activity of kaemferol 3-*O*-glucoside (not listed in Table 2) is similar to that of kaemferol and that of the C-glucoside orientin similar to that of its nonglycoside luteolin.

The most effective inhibitors of aldose reductase were the pentahydroxy flavones and their derivates (Table 3). Among the aglycones, quercetin was found to be the most potent. It inhibited the activity by 60 per cent at 10^{-6} M and retained a significant inhibitory activity at 10^{-7} M (15 per cent). The flavanone of quercetin (2,3-dihydro quercetin), known also as taxifolin, was less potent than the quercetin itself, showing again in this series that the presence of 2:3 unsaturation enhanced the inhibitory activity. Another feature conducive to inhibitory activity more definitely demonstrated in this series than in earlier ones was in the catechol orientation of hydroxyls in ring C. Morin, a pentahydroxy flavone with resorcinol (meta) type of orientation of hydroxyls, was much less potent than quercetin, where the hydroxyl groups have a catechol (ortho) orientation. In addition to the state of saturation of the ring B and the orientation of the hydroxyls in ring C, another feature significantly affecting the inhibitory activity was glycosylation. Both the position of the hydroxyls glycosylated and the glycosylating carbohydrate seemed to be important. Rutin (quercetin-3-*O*-rutinoside), in which the 3—OH is glycosylated by the disaccharide rutinose, was significantly less potent than the parent aglycon quercetin. On the other hand, the inhibitory activities of isoquercitrin (quercetin-3-*O*-glucoside) and hyperoside (quercetin-3-*O*-galactoside), the glycosides derived from monosaccharides, were only slightly different from the parent flavonoid. However, the inhibitory activity of quercetin-3-*O*-L-rhamnoside (quercitrin) was one order of magnitude greater than that of quercetin. This glycoside inhibited the enzyme activity by about 50 per cent at 10^{-7} M. The inhibitory activity was further enhanced if the second hydroxyl of the rhamnose moiety was acetylated. This compound, quercitryl 2″-acetate, inhibited the enzyme by 87 per cent at 10^{-7} M and 50 per cent at 4×10^{-8} M. Thus, this compound is the most potent inhibitor of aldose reductase known thus far. It was first isolated from the red wing azelia flower by Asen *et al.* [26] and characterized by Asen and Horowitz [27]. A similar acetate of quercetin-3-*O*-glucoside (isoquercitrin) could not be obtained but it was surprising that the activities of isoquercitrin and a compound isolated from the heavenly morning glory flowers tentatively identified as the potassium salt of

Table 1. Inhibition of lens aldose reductase

Structure	Name	Inhibition % M 10^{-4}	10^{-5}	10^{-6}	10^{-7}
Di and Trihydroxy Flavones					
	5, 7, -dihydroxy Flavone (Chrysin)	80	68	12	0
	Pinoembrin Rhamnoglucose (Saratonoside)	30	0	0	0
	4', 5, 7-Trihydroxy Flavone (Apigenin)	98	90	46	0
	4' Methoxy Apigenin	85	65	28	0
	Apiin	90	65	0	0
	Naringin	80	59	0	0

* Figures represent percentage inhibition of enzyme activity by the compounds under study as compared to controls run simultaneously in the absence of inhibitors. The enzyme activity was determined by the rate of decrease in $O.D._{340}$ due to NADPH utilization, monitored on a Gilford 2400-S recording spectrophotometer. The control reaction was carried on in a cuvette containing 0.1 M phosphate buffer, pH 6.2; NADPH, 2.5×10^{-4} M; DL-glyceraldehyde, 1.5×10^{-3} M; and the enzyme. The total volume of the reaction mixture was 1 ml. The reference blank consisted of all the above components except the substrate. The effect of inhibitors on enzyme activity was determined by including in the mixture described above the compound under study at desired concentrations. An appropriate blank to correct for absorption by the compounds was also run simultaneously. The reaction was carried on at room temperature. Each compound was tested four to six times and the deviation from the figures mentioned was less than 5 per cent. Pinoembrin was supplied by Dr. M. F. Refozo, Retina Foundation, Boston. Naringin was supplied by Alcon Laboratories, Fort Worth, Tex.

isoquercitrin acylated with malonic acid [28] were similar. It is apparent from these studies that minor modifications in the structure of sugar moieties can affect the inhibitory activity in a rather unpredictable way.

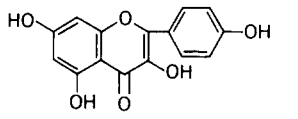
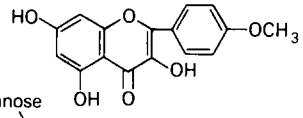
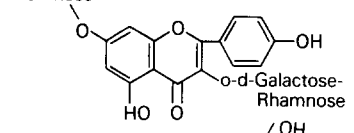
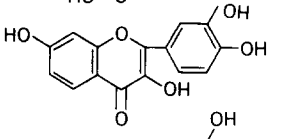
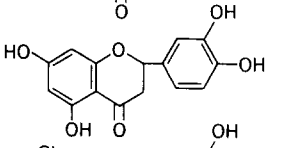
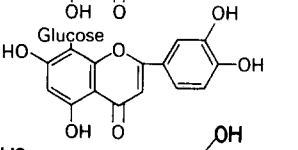
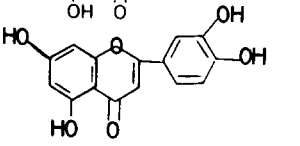
The addition of a sixth hydroxyl group to the pentahydroxy flavone in ring C compromises the activity to some extent. The inhibitory activity of myricetin, the hexahydroxy flavone, is somewhat less than that of quercetin (Table 4). That glycosidation at the 3—OH by L-rhamnose enhances the inhibitory activity is again demonstrated in that myricitrin, the 3-*O*-L-rhamnoside, is more potent than the parent aglycone myricetin.

Gossypin, which is the 8-*O*-glucoside of 3,5,7,8,3',4'-hexahydroxy flavone, was found to be a poorer inhibitor than quercetin and myricetin. The corresponding glucuronide (not listed in Table 4), however, although less active than quercetin, was as active as myricetin. It is obvious that the effect of introducing an 8—OH in the flavone moiety on the inhibitory activity needs further investigation.

The activity of another hexahydroxy flavone, veneen, was also found to be less than the corresponding pentahydroxy compound quercetin. The decrease in the potency of this compound as compared to quercetin is, however, due to the loss of the high electron density (unsaturation) from the ring B as observed in earlier examples.

Some flavonoids when dissolved can give rise to corresponding chalcones rather easily. This involves the opening of the oxide ring B. It may happen *in vivo* as well. It was considered necessary, therefore, to check whether the chalcones retain the inhibitory activity demonstrated by the flavonoids. The inhibitory activities of hesperidin and its chalcone, and chlorogenic acid, which also can be considered as a chalcone, are summarized in Table 5. The inhibitory

Table 2. Tetrahydroxy flavones

Structure	Name	Inhibition Percentage M			
		10^{-4}	10^{-5}	10^{-6}	10^{-7}
	3, 4', 5', 7 Tetrahydroxyflavone (Kaemferol)	87	62	0	0
	Kaemferide	90	50	0	0
	Robinin	100	56	0	0
	Fisetin	100	83	50	0
	2, 3-Dihydroluteolin (Eriodictyol)	90	60	20	0
	Orientin	90	82	45	0
	Luteolin	90	85	45	0

* Figures represent the percentage of inhibition as compared to controls run simultaneously. The details of the reaction, number of experiments and standard deviations were similar to those described in the legends to Table 1. Eriodictyol was supplied by Coopers Laboratories, N.J. Orientin was supplied by Drs. A. B. Segalman, Rutgers University, N.J. and S. Asen of U.S.D.A. Luteolin was supplied by Dr. Sam Asen, Dept. of Agriculture Research, U.S.D.A., Beltsville, Md.

activities of hesperidin and its chalcone are similar, indicating that the compound's activity is not lost when the B ring is opened. Chlorogenic acid is in fact very potent since it inhibits the enzyme activity by 25 per cent at a concentration as low as 10^{-7} M. These compounds, however, are more susceptible to oxidation and therefore less suitable than flavones.

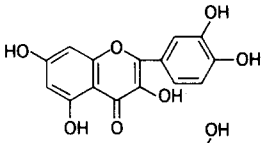
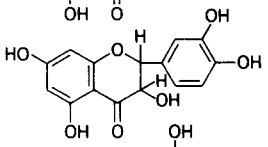
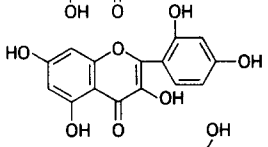
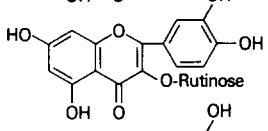
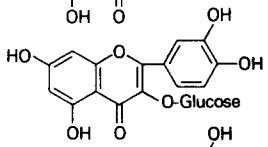
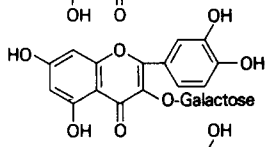
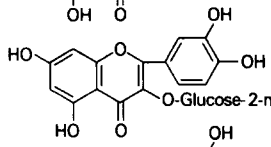
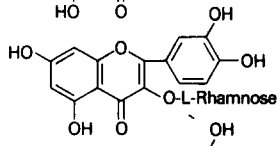
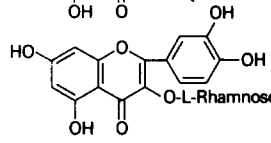
The inhibitory activity was also found to be associated with isoflavones (2-carbethoxy-5,7-dihydroxy isoflavone), catechins, and anthocyanins but they are much less potent than flavones (Table 6).

The coumarins, which are biogenically and chemically related to flavonoids, were also found to inhibit aldose reductase significantly. Esculetin (6,7-dihydroxy coumarin) inhibits the enzyme to about 50 per cent at 10^{-6} M (Table 7). This is close to the inhibitory activity of quercetin. Its glucoside, esculin, formed by glucosylation of the 6—OH, which corresponds to the 6—OH of the ring A of flavone, is much less potent. It inhibits the enzyme by only 15 per cent at 10^{-5} M. This pair of compounds thus further emphasizes the importance of catechol orientation in the inhibition of aldose reductase.

One of the serious limitations to the use of flavonoids for any therapeutic activity has been their insolubility in water. We therefore examined the inhibitory effects of some commercially available water-soluble derivatives synthesized by the Zyma Co. (Nyon). The structures of those compounds along with their inhibitory activities are described in Table 8. It was observed that these derivatives are much less potent than their parent natural analogues. The substitution of phenolic hydroxyls by ethanolic hydroxyls, which tend to increase the solubility, is accompanied by a decrease in the potency. The inhibitory activities of the various Tris derivatives occur in the following sequence: quercetin > mono-7-HEQ > rutin > mono-7-HER > Tris HER > tetra HER.

Kinetic studies were conducted with quercitrin to determine the type of inhibition exhibited by the flavonoids. The inhibitory action of quercitrin was also compared with that of AY-22,284 (an isoquinoline

Table 3. Pentahydroxy flavones

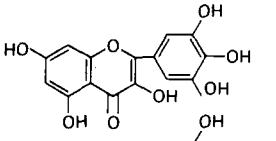
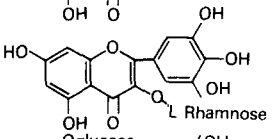
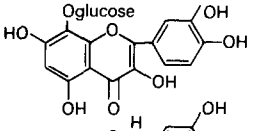
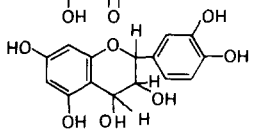
Structure	Name	Inhibition Percentage M			
		10^{-4}	10^{-5}	10^{-6}	10^{-7}
	3, 5, 7, 3', 4', Penta Hydroxy Flavone Quercetin	100	83	60	15
	3, 5, 7, 3', 4', Penta Hydroxy Flavanone Dihydro Quercetin	87	62	17	0
	Morin	100	75	0	0
	Rutin	95	95	20	10
	Isoquercitrin	95	70	45	0
	Hyperoside	95	68	50	0
	Isoquercityl-2-'' malonate	97	72	50	0
	Quercitrin	100	95	88	55
	Quercityl-2-''* acetate	100	100	100	87

* Figures represent the percentage of inhibition as compared to controls. The details of the reaction, number of experiments and standard deviations were the same as in Table 1. Quercetin and quercitrin were supplied by Alcon Laboratories, Fort Worth, Tex. Isoquercitrin and hyperoside were supplied by Prof. H. Wagner, University of Munchen, Germany, and by Dr. A. G. R. Nair, Medical College, Pondicherry, India. Synthetic isoquercitrin was made available through Alcon Laboratories, Fort Worth, Tex. Quercitryl acetate and isoquercitryl malonate were extracted and purified by Dr. Sam Asen of the Dept. of Agriculture Research, U.S.D.A. Beltsville, Md. Isoquercitrin was also supplied by Dr. W. B. Mors, University of Rio de Janeiro, Brazil. Also known as 2''-*O*-acetyl quercitrin.

derivative), the earlier most potent and extensively studied aldose reductase inhibitor. Figure 1 is the Lineweaver–Burk plot of the kinetic data. The K_m of the reaction was found to be unaffected either by quercritin or AY-22,284, while the V_{max} decreased in both cases. The inhibition of aldose reductase by both compounds is thus of the non-competitive type. The K_i for quercitrin was 1.2×10^{-7} M against 1.6×10^{-6} M for AY-22,284; by this criterion, quercitrin is at least one order of magnitude more potent than AY-22,284.

We next tested the potent inhibitors in the intact lens in organ culture using xylose. Xylose is a good cataractogenic sugar to use, since it is rapidly converted to xylitol by aldose reductase as compared to the conversion of glucose to sorbitol and of galactose to dulcitol. The results of this series of experiments have been summarized in Fig. 2. At 10^{-4} M quercitrin

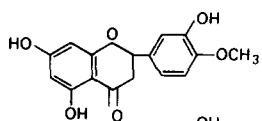
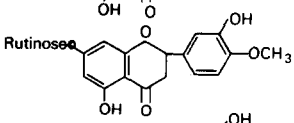
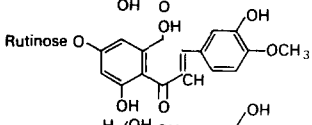
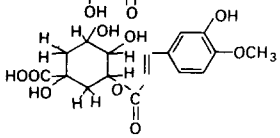
Table 4. Hexahydroxy flavones

Structure	Name	Inhibition Percentage				
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	M
	Myricetin	100	55	32		
	Myricitrin	100	100	75	35	
	Gossypin	90	40	0	0	
	Veneen	60	25	0	0	

* Details of reaction, number of experiments and standard deviations were similar to those in Table 1. The numbers indicate per cent inhibition of enzyme activity as compared to controls. Gossypin was supplied by Mr. N. S. Parmar of Pondicherry Medical College, India. Veneen was supplied by Alcon Laboratories, Fort Worth, Tex.

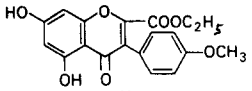
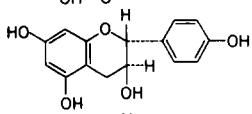
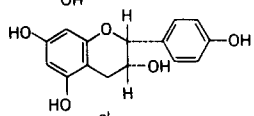
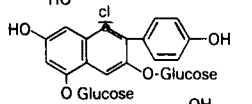
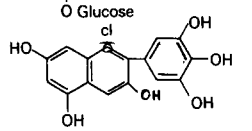
decreased the polyol accumulation by 80 per cent of the control, a value which was double the decrease brought about by AY-22,284. It was further significant that the polyol accumulation was reduced noticeably at 10^{-5} and 10^{-6} M concentrations of quercitrin, AY-22,284 being ineffective at these levels. However, quercitrin was less effective against the enzyme in the intact lens than against the partially purified enzyme. This may have been due to the low degree of permeability of quercitrin or due to other unknown factors like metabolism of the flavonoid, its cellular compartmentalization or other interactions. A comparison of the culture experiments with enzyme determination experiments is thus not possible at the present time. Nevertheless, under culture conditions also, quercitrin was more effective than AY-22,284. Quercityl-2″-acetate was even more effective. At 10^{-5} M it inhibited polyol accumulation by 58 per

Table 5. Hesperetins and chlorogenic acid

Structure	Name	Percentage Inhibition M			
		10^{-4}	10^{-5}	10^{-6}	10^{-7}
	3', 5, 5-Trihydroxy -4'-Methoxy Flavanone Hesperetin	100	59	0	0
	Hesperidin	66	10	0	0
	Hesperidin Chalcone	82	10	0	0
	Chlorogenic Acid	95	90	78	25

* Figures represent the percentage of inhibition as compared to controls. The experimental details, number of experiments and standard deviations were similar to those described in Table 1. All samples were obtained commercially.

Table 6. Isoflavone, catechins and anthocyanins

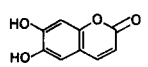
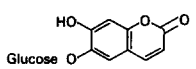
Structure	Name	Percentage Inhibition M			
		10^{-4}	10^{-5}	10^{-6}	10^{-7}
	2-Carbethoxy-5, 7 Dihydroxy 4′ Methoxy Isoflavone	100	77	0	0
	D-Catechin (2R:3S)-5, 7, 3′, 4′-Tetrahydroxy Flavan-3- 1	50	10	0	0
	L-Catechin (2R:3R) 5, 7, 3′, 4′ Tetrahydroxy Flavan-3-o1	56	25	20	0
	Pelargonin 3, 5-Bis (Glucosyloxy)-4′, 7 -Dihydroxy Flavylium Chloride	75	0	0	0
	Delphinidin Chloride 3, 3′, 4′, 5, 5′, 7 -Hexahydroxyflavilium Chloride	87	29	0	0

* Figures represent the percentage of inhibition as compared to controls. The experimental details, number of experiments and standard deviations are described in the legend to Table 1. Catechins were obtained from Zyma (Nyon).

cent. Thus, the inhibitory activity of this compound was the highest of all the compounds studied. Overnight pre-incubation of lenses with quercitryl-2″-acetate at 10^{-5} M gave about 85 per cent inhibition. Thus, it seems to be a very promising analogue.

To determine whether flavonoids have undesirable effects on the lens, their action on overall metabolic parameters was examined. The results of the rubidium uptake experiments which measure the ion pump activity of the tissue indicated that quercitrin does not have any effect on this process, the ratio of the concentration of ^{86}Rb in lens water with respect to that in the medium being 20 ± 1 in both the absence and the presence of the inhibitor (10^{-4} M) in the medium of incubation. The amount of lactate present in the lenses incubated in the control medium was 16 ± 1.0 μg/lens. This value remained unchanged in the presence of quercitrin. In addition, the flavonoid did not affect the ATP level of the tissue (2.45 ± 0.23 μmoles/g wet wt). These metabolic parameters were unaltered by quercetin also. Values de-

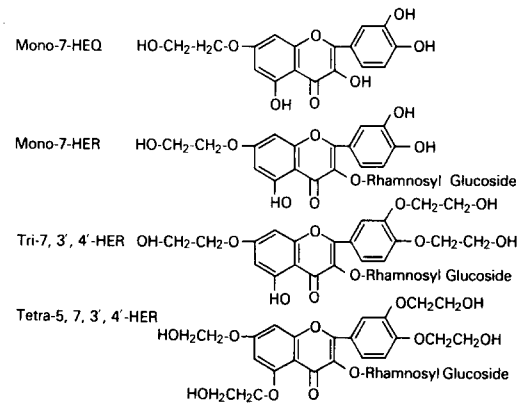
Table 7. Coumarins

Structure	Name	Percentage Inhibition M			
		10^{-4}	10^{-5}	10^{-6}	10^{-7}
	Esculetin 6, 7-Dihydroxy Coumarin	91	75	53	19
	Esculin 6, 7-Dihydroxy Coumarin 6-Glucoside	70	15	0	0

* Figures represent the percentage of inhibition as compared to control. The experimental details were the same as those described in the legend to Table 1.

Table 8. Inhibition of lens aldose reductase by Zyma flavonoids

Concentration (M)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Compounds					
Mono-7-HEQ	100	95	75	30	0
Mono-7-HER	50	30	10	0	0
Tri-7, 3′, 4′-HER	30	20	12	0	0
Tetra-5, 7, 3′, 4′-HER	25	0	0	0	0



* Figures represent percentage of inhibition as compared to controls. The experimental details were the same as those described in Table 1. The compounds were supplied by Zyma (Nyon). The chemical names of the compounds are as follows: mono-7-HEQ, 7-*O*-(β-hydroxyethyl)-quercetin; mono-7-HER, 7-*O*-(β-hydroxyethyl)-rutin; tri-7,3′,4′-HER, 7,3′,4′-tri *O*-(β-hydroxyethyl)-rutin; and tetra-5,7,3′,4′-HER, 5,7,3′,4′-tetra-*O*—(β-hydroxyethyl)-rutin.

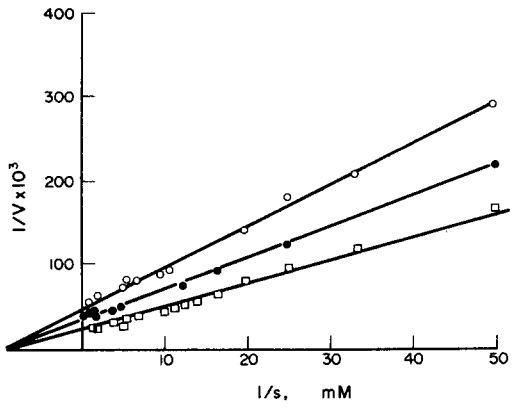


Fig. 1. Lineweaver–Burke plot: (□——□) control, (●——●) in the presence of AY-22,284 10^{-6} M and (○——○) in the presence of quercitrin 10^{-7} M. The velocity of aldose reductase reaction was measured for 5 min at each substrate concentration, in the presence and absence of inhibitors. The substrate was DL-glyceraldehyde. The reactions were carried on as described in Table 1 except that the glyceraldehyde concentration was varied. The enzyme activity was such that it resulted in a decrease of about 0.03 O.D. units/mg of protein/5 min of the reaction time at a substrate concentration of 1.5×10^{-3} M; $v = \Delta O.D._{340} \times 1000/\text{mg}$ of protein/5 min of reaction time.

scribed above represent the means (± standard deviation) of at least six experiments.

DISCUSSION

The results indicate that flavonoids as a class inhibit the enzyme aldose reductase to varying degrees. Many inhibit the enzyme by about 50 per cent at concentrations as low as 10^{-6} or 10^{-7} M indicating that these flavonoids are at least as potent as AY-22,284 or TMG (3,3′-tetramethylene glutaric acid), previously considered the best aldose reductase inhibitors. AY-22,284 and TMG inhibit the enzyme by 40 per cent at 10^{-6} M. Thus, quercitrin and quercityl acetate are at least 13–25 times more potent than the inhibitors previously known. The inhibitions appear to be noncompetitive in nature, a factor preferable in therapeutic use over competitive inhibitors.

Previous studies indicate that structural requirements of an aldose reductase inhibitor are more general than specific. However, the common feature of all such inhibitors is a hydrophobic region attached to an acid group. The flavonoids meet these requirements. Although the inhibitory activities of various flavonoids differ from each other, it is not possible to define a clear relationship between the structure and inhibitory potency of the compound. However some possible inferences are summarized as follows: (1) flavones are more potent than flavanones; (2) ethers are less potent than phenols; (3) glycosylation of ring A, particularly of the 7—OH by a disaccharide, leads to a decrease in the inhibitory activity; (4) C-glucosidation in ring A does not affect the potency; (5) in the flavonols, an orthodihydroxy (catechol) orientation in ring C (3′,4′-dihydroxy) is more conducive to the inhibitory property as compared to metadihydroxy (2′,4′-dihydroxy) orientation; (6) 3-*O*-glycosylation with a disaccharide decreases the inhibitory activity; (7) 3-*O*-glycosylation with D-glucose or D-galactose is without any effect, 8-*O*-glucosylation is detrimental; (8) 3-*O*-glycosylation with L-rhamnose increases the inhibitory activity; (9) acylation of the secondary alcohol group with a monocarboxylic acid increases the potency further; acylation with a dicarboxylic acid is without any effect; (10) increasing the number of —OH groups in ring C to two enhances the inhibitory activity, but additional —OH groups in ring C are slightly depotentizing; (11) the inhibitory activity of chalcone is of the same order as of the corresponding flavanone; (12) a ketone group at C_4 is required since anthocyanins and catechins are poor inhibitors; and (13) phenolic hydroxyls are preferred over alcoholic hydroxyls in the flavonoid moiety.

One of the difficulties with the use of flavonoids is their aqueous insolubility. From this point of view, two interesting compounds discovered were quercetin-3-*O*-glucosyl-2-malonate and quercetin-3-*O*-rhamnosyl-2-acetate. These derivatives are completely water-soluble. The malonate derivative is found naturally in Morning Glory flowers and the acetate in Azelia flowers. Unlike synthetic water-soluble derivatives of the Zyma Co., which are much less potent than their parent compounds, these natural water-soluble derivatives are at least as active as the parent or even more. The acetate derivative of quercitrin was found to be the best inhibitor of aldose reductase known so far. Its water solubility and high potency are thus assets that may prove useful in the prevention of sugar cataracts in animals. Our preliminary studies indicate that flavonoids can prevent or at least delay the formation of diabetic cateracts in animals.

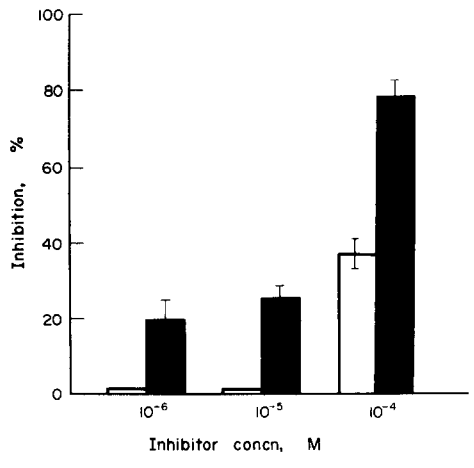


Fig. 2. Inhibition of xylitol synthesis in intact lens by quercitrin and by AY-22,284. Lenses obtained for 100 g rats were used. One lens was incubated in medium with inhibitor at the indicated concentration; the contralateral lens was incubated in medium without the inhibitor. The temperature of incubation was 37°. Xylose concentration in the medium was identical in both cases (30 mM). Each bar represented six to eight experiments. The figures represent the per cent decrease in xylitol accumulation due to inhibitor as compared to the contralateral controls. Values are means ± standard deviation. The amount of xylitol accumulated by control lenses during the period of incubation (4 hr) was 12 ± 0.65 nmoles/mg wet wt.

The results described herein suggest the possibility that there may be other flavonoids with more potent activity against aldose reductase and that the highly potent ones may be useful clinically. For this reason this survey is being continued.

It should be pointed out that although inhibition of aldose reductase is a consistent action of flavonoids, they have been reported to affect a number of other metabolic reactions. Danon and Elazar [29] observed that *O*-(β-hydroxyethyl)-rutins increase the rate of glucose utilization by red blood cells. Fritzniggli [30, 31] noted that bilirubin-suppressed oxidative phosphorylation in rat liver mitochondria is counteracted by hydroxyethyl rutinosides. These compounds were also found to attenuate or counteract the detrimental effect of aging, irradiation and iodoacetamide on oxidative phosphorylation in isolated liver mitochondria. Capelli *et al.* [13, 14] reported that rutins added to tissue cultures of chick arteries slow the degeneration of vessels by inhibiting the production of mucopolysaccharide. Disorders of the metabolism of muscle cell in culture as indicated by the appearance of mucopolysaccharide are also delayed by flavonoids [28]. Recently, Soulina *et al.* [9] have proposed that flavonoids can be used to decrease the rate of glycolysis, which was found to be accelerated in some types of cancerous tissues. It was considered that this is done by reducing the phosphate supply through inhibiting Na^+K^+-ATPase [8]. We, however, could not demonstrate any effect of flavones on ion pump activity or lactate production by lens. Nevertheless, the reports do indicate that flavonoids, commonly present in food, may affect cellular metabolism in several ways. The mechanism of such diverse action, however, is poorly understood. Since the physiological role of aldose reductase is not well known, it is difficult at the present time to speculate on the role of flavonoids in normal cellular metabolism, but in hyperglycemic stress they would perform the useful role of preventing hydration and edema of certain tissues caused by polyol accumulation. This, in turn, will prevent or delay the formation of sugar cataracts and ameliorate against other possible manifestations initiated through polyols. The flavonoids, therefore, may have a useful role in diabetes.

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