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ASSAY OF THE CAPILLARY ACTION OF FLAVANOID COMPOUNDS IN MICE

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A method is presented for the assay of biologically active flavanoids by measurement of the reduction which these compounds cause in the sensitivity of the skin capillaries of mice to histamine. The assay is economical of time, space, and apparatus. Limits of error may be calculated from internal evidence. Time/effect relationships are also given for the capillary actions of rutin and hisperidin and for some of their derivatives.

The capillary actions of biologically active flavanoid compounds have hitherto been assayed in rabbits and in guinea-pigs. Three different criteria have been used for the measurement of the intensity of their action in these species. First, Bacharach, Coates, and Middleton (1942) used the effect of these compounds on the tensile strength of capillary walls. They measured the negative pressure required to produce petechial haemorrhages in guinea-pigs fed on a scorbutogenic diet supplemented with cod-liver oil and a known weight of ascorbic acid. Flavanoid compounds, administered orally, increased capillary strength. One month of treatment was needed before maximum discrimination between dose levels developed. The next criterion used was the antagonism by flavanoid compounds of the local increase in capillary permeability which results from irritation of the skin. Ambrose and de Eds (1947) made application of chloroform for 30 sec. to the depilated skin of rabbits, and Bohr, McIvor and Rinehart (1949) applied a brass plate at 58° for 30 sec. to the hairless skin of guinea-pigs to produce local irritation. Decrease in the resulting local change in permeability was measured as an increase in the time before coloration, due to leakage of dyes from the blood stream, appeared at the site of cutaneous irritation. Finally, Schiller (1951) made the potentiation caused by flavanoid compounds in the cutaneous vasoconstrictor actions of adrenaline and noradrenaline the basis of an assay in rabbits.

The assay method described here employs mice, and may be regarded as a modification of the rabbit method of Last and Loew (1947) for the assessment of antihistamine activity. The advantages of this mouse method are speed, economy of space, and simplicity. In addition, limits of error are calculable from the internal evidence of each assay.

METHODS

Male mice, weighing 20 to 35 g., varying by not more than 5 g. in each experiment, were used in groups of 6, 8, 9, or 10. Three different strains of white mice were used in the course of the work.

Flavanoid Compounds.—Rutin (Allen and Hanbury), Hisperidin, Hisperidin Methyl Chalcone, Quercitin, Naringin (a gift from Beecham Food Group), and Chalcone (Kodak) were dissolved in propylene glycol (B.D.H.). Volumes not exceeding 0.2 ml./mouse were injected by the intraperitoneal, and 0.075 ml. by the intravenous, routes.

The Dependence of Effect on Dose.—A separate group of mice was assigned to each dose level. A control group was assigned to each solvent volume. Each mouse in each group was treated as follows: (*a*) Test solution or solvent was injected at zero time. (*b*) After a fixed time interval (usually 20 to 30 min. for the intraperitoneal, and 15 to 20 min. for the intravenous, assay route) 0.15 ml. of pentobarbitone sodium solution containing 12 mg./ml. in aqueous 2% ethyl alcohol and 4% propylene glycol was injected intraperitoneally. (*c*) During 8 to 10 min. after (*b*), four well-separated intradermal injections of 1 μg. histamine acid phosphate in 0.02 ml. of 0.9% NaCl were made into the abdominal skin. (*d*) Ten minutes after (*b*), 0.1 ml. of a filtered 1% solution of Evans Blue in 0.9% NaCl was injected into the tail vein. (*e*) Fifteen minutes after (*d*), the mouse was rapidly killed. (*f*) Within two hours of death, the abdominal skin flap was resected and turned over so that the inner side lay uppermost. The depth of the blue colour was scored for each injection site by reference to a colour density chart. The total score for each

mouse was recorded. Colour density charts were made from smooth white paper, with 0.015% Evans Blue in water (w/v) and a paint brush. Successive washes of colour were applied to the paper, which was allowed to dry between each wash. Colour matching a single wash scored 1, a double wash scored 2, a treble wash scored 3, etc. The score range 3 to 9 was that suitable for use.

Variation of Effect with Time.—Four to six groups of 6 mice were injected with a fixed dose of test compound in a fixed volume of solvent; the same number of groups were injected with the solvent alone. Treatments (*b*) to (*f*) were applied to one test group and to one solvent group starting 1, 10, 20, 30, 40, and 60 min. after the initial injection.

Assay of the Capillary Action of Flavanoids.—Groups of not less than 9 mice are recommended. Two doses of standard (rutin) and two of test compound, which produced a submaximal, measurable, and comparable effect, were selected. The ratio of the high dose to the low dose was the same for standard and test compounds, and was normally 2.0. High doses were administered in 0.2 ml. and low doses in 0.1 ml. of propylene glycol intraperitoneally. Six groups of mice were required; one for each dose level of standard, of test, and of solvent. The effect of these doses on the resistance of the skin capillaries to the action of histamine was measured at the time of their maximum activity. This time had, in each case, been determined by previous experiment.

When no significant difference was found between either the mean score for each dose of test or of standard solution and the mean score for the corresponding solvent volume, the assay was discarded, and doses were readjusted before its repetition. This last has been necessary in two out of eighteen assays.

The assay procedure was modified when aqueous extracts containing naturally occurring flavanoid compounds were compared for their capillary action. Rutin in propylene glycol was again used as standard. Two additional groups of mice were injected with volumes of aqueous solvent corresponding to the volumes of test solution used. Differences between individual scores for mice treated with test and standard solutions and the mean score for mice correspondingly treated with solvent were expressed as % of those means. The values obtained were subjected to statistical analysis as described by Schild (1942). Corrections for coarse grouping were not applied.

Results

Rutin was selected for use in preliminary studies of the action of flavanoid compounds on the resistance of the skin capillaries of mice to the permeability changes induced by the intradermal injection of histamine, for the following reasons. Firstly, it was reasonably soluble in propylene glycol. Secondly, it was readily available in pure form. Thirdly, the capillary actions of rutin had been examined by all the methods formerly used for the assay of vitamin-P-like activity, and it was intended to use rutin as the reference compound for such assays in mice.

The Dependence of the Intensity of the Action of Rutin on the Dose Administered by Intraperitoneal Injection.—Measurement was made of the extent to which rutin antagonized the local increase in capillary permeability caused by the intradermal injection of histamine 45 min. after the intraperitoneal injection of this flavanoid. The combined results of three experiments are shown in Table I. The solvent, propylene glycol, itself

TABLE I

DOSE/EFFECT RELATIONSHIP FOR THE ACTION OF RUTIN (ADMINISTERED INTRAPERITONEALLY TO MICE IN PROPYLENE GLYCOL) ON CAPILLARY RESISTANCE TO HISTAMINE

Rutin/ Mouse mg.	Solvent ml.		Mean Scores/Mouse ± S.E. (No. of Mice)	
	Propylene Glycol	Saline	Recorded	Difference from Control Solvent
5	0·1	—	16·5 ± 1·02 (18)	7·6 (propylene glycol)
10	0·2	—	10·4 ± 0·86 (18)	10·0 ,, ,,
20	0·2	—	7·9 ± 1·52 (18)	12·5 ,, ,,
—	0·1	—	24·1 ± 1·46 (18)	3·5 (saline)
—	0·2	—	20·4 ± 0·68 (18)	7·2 ,,
—	—	0·2	27·6 ± 0·74 (18)	—

caused some reduction in the responses of the vessels to histamine. This is made evident by comparison of the mean scores obtained after the intraperitoneal injection of the glycol and of 0.9% NaCl (w/v aqueous) into separate groups of mice. The effect of the propylene glycol alone was not, however, as great as when rutin was administered in it. The actions of rutin alone and of propylene glycol alone have been isolated in the last column of this Table. Both were graded in respect of dose, and that produced by rutin was linearly related to log. dose.

The use of propylene glycol as a solvent was made necessary by the limited solubility of many of the compounds under test. Its effects on the skin capillaries therefore received further study, and were finally attributed to the decrease in cutaneous blood flow and capillary pressure associated with its narcotic action. Increase in the depth of pentobarbitone anaesthesia similarly decreased the mean score/mouse in response to standard intradermal injections of histamine. The slopes of curves relating reduction in the histamine response to log. dose, given intraperitoneally, were very similar for these two narcotics. It was therefore theoretically possible to compute the reduction to be made in the dose of pentobarbitone for the use of a given volume of propylene glycol, such that the responses of the skin vessels should remain constant towards histamine.

In practice, however, the anaesthetic effects of propylene glycol were insufficient to allow of the necessary precision of high-speed intradermal injections. This finding made it necessary to limit the amount of propylene glycol used as solvent to 0.2 ml./mouse by intraperitoneal injection, and 0.075 ml./mouse by intravenous injection, and to include solvent controls in all quantitative experiments.

The dye, Evans Blue, was found to have capillary action. Intravenous injections of 0.1 ml. 1% Evans Blue made before intradermal injections of 1 μg. histamine acid phosphate in 0.02 ml. resulted in significantly less blue colour at the cutaneous injection sites than was found when the order of these injections was reversed (Table II). This made

TABLE II

THE EFFECT OF INTRADERMAL HISTAMINE ON SKIN PERMEABILITY IN THE PRESENCE OF EVANS BLUE

Time of Injection of Dye	Mean Score/Mouse±S.E. (No. Mice)
2 min. before histamine	14·3±2·86 (8)
1 ,, after ,,	26·8±0·76 (8)
2 ,, ,, ,,	27·2±1·32 (8)

it necessary to control the times of injection of both the histamine and the dye strictly during quantitative work. First, intradermal injections of histamine were made in a time interval not exceeding 40 sec. Then the Evans Blue was injected intravenously within the interval of 1 to 1.5 min. after the last histamine injection.

Post-mortem examination of mice which had received intraperitoneal injections of rather insoluble flavanoids dissolved in propylene glycol 1 hr. previously, for example hisperidin, frequently revealed the presence of finely divided flavanoid precipitated in the peritoneal cavity. Since the intraperitoneal injections of suspensions of talc in 0.9% NaCl were without effect on the responses of the cutaneous vessels to intradermal injections of histamine, it was concluded that the most accurate possible method of expressing the activity of flavanoid compounds administered by the intraperitoneal route would result from measurement both of the intensity and duration of their action. In practice, time/effect relationships have been determined at a single dose level, and potency has been measured at the time of maximum activity. This procedure was adopted for economy of test material, mice, and time.

Finally, since this rapid assay method was likely to find its use in assisting the isolation of naturally occurring substances with vitamin-P-like action from vegetable extracts, the possibility that well-recognized groups of organic compounds might interfere with the responses of skin capillaries to histamine was briefly reviewed. The intraperitoneal, but not the intravenous, injection of glucose, fructose, and cane sugar caused the gradual onset of a period of decreased sensitivity of the skin vessels to histamine. This change was demonstrable in 15 min., reached maximum at 30 to 40 min., and had decreased but was still significant at 60 min. The intensity of change appeared to be more closely related to the total amount of sugar injected than to the tonicity of the solution used. The magnitude of this change is illustrated by the results of a typical experiment in Table III.

TABLE III

THE EFFECT OF GLUCOSE AND FRUCTOSE SOLUTIONS, INJECTED IN A VOLUME OF 0·4 ML. INTRAPERITONEALLY, 30 MIN. BEFORE TESTING SENSITIVITY OF SKIN CAPILLARIES TO HISTAMINE

Solution Injected Intraperitoneally	Mean Score/Mouse
Saline	25·8±3·6 (6)
Glucose 6% in water	22·3±2·4 (6)
,, 6% in saline	22·7±2·1 (6)
Fructose 6% ,,	22·5±3·1 (6)
Glucose 12% in water	16·7±1·9 (6)
,, 12% in saline	16·2±2·3 (6)
,, 6% and fructose 6% in water	16·3±2·4 (6)
Saline	25·4±1·9 (6)

The Dependence of the Action of Flavanoid Compounds on the Weight Administered Intravenously.—The intravenous injection of 10 mg. rutin or of 5 mg. hisperidin in 0.15 ml. propylene glycol into the tail veins of mice was immediately followed by cyanosis. Microscopic examination of the lungs and mesentery showed that many capillaries became blocked by precipitated flavanoid within 2 min. of the injections. This observation made worthless estimates of the capillary actions of flavanoids characterized by a low solubility in water and administered intravenously.

Aqueous extracts containing water soluble flavanoid glycosides from various fruits caused reductions in the sensitivity of skin vessels to histamine which did not appear for 10 to 20 min. after their intravenous injection. The intensity of the capillary action of these extracts was, when fully developed, related linearly to log. dose.

The Relationship between Intensity of Action of Flavanoid Compounds on the Skin Capillaries and Time after Administration.—The delayed onset of the capillary actions of water soluble flavanoid compounds after intravenous injection indicated that changes brought about in the chemical structure of these compounds by metabolic processes conferred or greatly enhanced their capillary actions. Time/effect curves of flavanoid compounds of known structure were therefore studied

for the purpose of discovering, within the limitations of the meagre series available, the effect of change in chemical structure on this latent period.

The structural unit common to all flavanoid compounds is 2-phenyl benzopyrone. Most of those found in fruit are glycosides in which the sugars, usually glucose and rhamnose, are attached to the aglycone in positions 7 and 3. Three commonly occurring types of flavanoid compounds differ in their structure at position 3; these are the flavones, the flavanols, and the flavanones. The flavones were represented by the glycoside rutin and its aglycone quercitin in the series studied. The glycosides hisperidin and naringin represented the flavanones. None of these glycosides had sufficient solubility in water to permit measurement of the latent period of their capillary action by intravenous injection. They were given dissolved in propylene glycol by the intraperitoneal route. Two chalcones, hisperidin methyl chalcone derived from a flavanone, and chalcone derived from the flavone rutin, were also examined. These compounds and also quercitin were sufficiently soluble to be studied both by intravenous and by intraperitoneal administration.

Time/effect relationships for all these compounds are shown in Table IV. Reduction in the sensitivity of skin capillaries to histamine appears in this Table as a reduction in the mean score/mouse for the blue colour which leaked from the capillaries at the sites of intradermal injections of histamine. The time course of the action of the flavone glycoside rutin resembled very closely that of the flavanone glycoside hisperidin. Capillary action did not appear for 20 min. and developed to a maximum in 40 min. after the intraperitoneal injection of these compounds. The time course for the action of the aglycone quercitin was indistinguishable from that of the parent glycoside rutin administered by intraperitoneal injection, whether the quercitin was given by the same or the intravenous route. It was therefore concluded that sugar molecules attached to the aglycone neither hindered absorption from the intraperitoneal cavity nor delayed activation of the aglycone by metabolic processes. By contrast, reduction in the sensitivity of skin capillaries to histamine appeared within 10 min. of either the intraperitoneal injection or the intravenous injection of chalcones derived from both hisperidin and rutin. These chalcones, especially the highly purified methyl derivative of hisperidin, had intense but short-lived action by intravenous injection and only small activity when given into the peritoneal cavity. This indicated their breakdown to inactive metabolites in the liver. Finally the action of hisperidin methyl chalcone was shown to have reached maximum intensity within 3 min. of its intravenous injection. The evidence obtained from these time/effect studies was therefore compatible with the hypothesis that the glycosides and aglycones owe their capillary action to chalcones formed from them in the animal body.

Flavanoid Compounds Failed to Antagonize the Action of Histamine on Smooth Muscle.—All attempts to demonstrate inhibition of submaximal responses of the isolated guinea-pig ileum to histamine by hisperidin, rutin, naringin, and quercitin failed. Evidence has, however, been presented which strongly indicates that the antagonism shown by these glycosides and by the aglycone to the action of histamine on the capillaries of intact animals should be attributed to the formation of active intermediary compounds by metabolic processes (Table IV). Both hisperidin methyl chalcone and the chalcone developed their action

TABLE IV

ME EFFECT RELATIONSHIPS FOR THE ACTION OF SOME FLAVANOID COMPOUNDS ON THE RESISTANCE OF SKIN CAPILLARIES OF MICE TO HISTAMINE

Correction has been made for solvent action in the compilation of this table.

Compound		Route of Injection	Dose/Mouse mg.	Mean Score/Mouse ± the Standard Error (No. of Mice)				
				Control	Time in Min. After Injection			
Name	Nature				10 to 15	20 to 25	30 to 40	55 to 65
Rutin	Glycoside	I.P.	10	20·7±1·26 (10)	19·8±1·34 (10)	16·7±2·58 (10)	13·8±2·13 (10)	13·2±1·78 (10)
Hisperidin	,,	,,	10	21·3±1·08 (10)	21·0±1·27 (10)	15·1±1·76 (10)	12·3±1·82 (10)	12·6±1·44 (10)
Naringin	,,	,,	10	20·4±2·12 (6)	21·3±1·78 (6)	20·7±1·63 (5)	21·2±2·14 (5)	—
Quercitin	Aglycone	,,	10	21·2±1·37 (6)	20·5±2·16 (6)	18·8±2·14 (6)	14·2±2·36 (6)	14·0±2·79 (6)
		I.V.	5	22·9±1·32 (6)	21·8±1·12 (6)	16·4±1·51 (6)	—	—
Hisperidin methyl chalcone	Chalcone	I.P.	5	18·8±1·24 (6)	17·5±0·95 (6)	17·7±1·29 (6)	17·8±1·51 (6)	18·0±1·41 (6)
		I.V.	5	20·7±0·55 (6)	4·5±2·64 (6)	—	15·0±1·61 (6)	—
		,,	2·5	21·9±0·27 (6)	9·0±3·13 (6)	14·8±2·38 (5)	—	—
"Chalcone" (Kodak, Ltd.)	,,	I.P.	10	20·8±2·32 (6)	17·8±2·11 (6)	15·4±1·79 (6)	16·8±2·32 (6)	—
		I.V.	10	21·7±3·31 (5)	12·2±2·24 (6)	14·3±1·70 (6)	—	—
		,,	10	20·6±1·78 (6)	11·5±2·17 (6)	—	—	—

in the whole animal immediately after they had been injected intravenously (Table IV). These compounds therefore required no activation. They were tested for ability to antagonize the action of histamine on smooth muscle both in isolated preparations and in the whole animal. The isolated preparations used were guinea-pig ileum, uterus, and tracheal chain ; these were suspended in oxygenated Tyrode fluid at 32°. Neither chalcone, when added to the bath fluid in concentrations up to 0.1 mg./ml. for periods of contact with the tissue up to 20 min., modified the responses of the tissues to histamine. The intravenous injection of 45 mg./kg. of either chalcone into guinea-pigs anaesthetized with pentobarbitone also failed to alter the pressor responses of the mean arterial pressure to histamine given intravenously. Observations were maintained for 40 min. after the injection of the flavanoid compounds in this last group of experiments.

It was therefore concluded that these flavanoid compounds had no true selective antihistamine action at the dose levels used and that they had antagonized the effects of histamine on the capillaries of the skin solely by their non-specific action on capillary permeability. This type of action was studied by previous authors ; it has been termed, by them, "vitamin-P-like" activity (Bacharach *et al.*, 1942 ; Bourne, 1943 ; Bacharach and Coates, 1944).

The Assay of Flavanoid Compounds for their Capillary Action in Mice.—Both pure flavanoid compounds and extracts of natural products which contained them have been assayed by measurement of the reduction they caused in the sensitivity of the skin capillaries of mice to histamine.

Sixteen 2 by 2 assays have been performed in which rutin in propylene glycol, 50 mg./ml., was used as standard and both standard and test solution were given by intraperitoneal injection. The mean slope of the dose/effect curve in these assays was 15.48. The standard error of this slope, directly determined, was 0.96. Both the number of mice used on each point and the strain of mice used appear to have influenced the precision of these assays. Table V summarizes these observations. In the first seven assays there were 6 mice in each group ; mice from British Drug Houses were used for three and mice from Glaxo Laboratories for four of these assays. Values of b and of s/b have been calculated for each assay. The mean slopes (b) of the dose/effect curves given by the two strains of mice were very similar, but the precision of the assays (s/b) was greater when the B.D.H. strain was employed. The use of 9 or 10 mice in each group further improved the precision of these assays. Overall, irrespective of the strains of mice used, the limits of error ($P=0.95$) obtained with 6 mice in a group averaged $\pm 39.8\%$ of the mean ; the standard error of this average was $\pm 3.11\%$. In the assays in which either 9 or 10 mice were used on each point, the limits of error ($P=0.95$) averaged $\pm 18.93\%$ of the mean, and the standard error of the average was $\pm 2.55\%$. Significant deviation from parallelism of the regression lines for test and standard solutions was not encountered.

TABLE V

VARIATION IN THE PRECISION OF 2 BY 2 ASSAYS OF THE CAPILLARY ACTION OF FLAVANOID COMPOUNDS IN MICE OF DIFFERENT STRAINS

No. of Mice/ Group	No. of Assays	Strain of Mice	Regression b±S.E. (No. Assays)	Range of s/b
6	7	Glaxo	15·1±2·7 (4)	0·32 to 0·51
		B.D.H.	15·3±2·5 (3)	0·17 o 0·35
9 or 10	9	,,	15·7±1·9 (8)	0·07 to 0·21
		Roche	15·8±3·7 (1)	0·19

DISCUSSION

The method presented for the assay of the capillary actions of flavanoid compounds in mice requires reasonable skill and careful attention to timing. The advantages of this method are believed to be speed and economy in space and apparatus. In addition, limits of error are calculable from the internal evidence of each assay.

It may be argued that the doses of flavanoid compounds needed for the purpose of rapid assays (such as 10 mg./30 g. mouse) were so large that these compounds cannot possibly have biological significance. However, their cumulative action has been demonstrated by Bacharach *et al.* (1942), and some fruits are very rich in these compounds. Bacharach and Coates (1944) measured the vitamin-P-like activity of citrus fruits, rose hips, and blackcurrants, and also that of concentrates and purées prepared from these fruits. Their richest source of activity was the peel of citrus fruit. Blackcurrants had the highest potency when this was measured in terms of fresh whole fruit. One hundred grammes of blackcurrants had capillary action equivalent to that of 750 mg. hisperidin. Purées made from this fruit developed further activity in the process and this activity was not destroyed by two years of storage at 0°. These purées developed activity equivalent to 5 g. hisperidin/100 g. of fruit. The possible biological

activity of such natural products cannot therefore be ignored.

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