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CHAPTER I

INTRODUCTION

The flavonoid compounds comprise a rather large class of naturally occurring phenolic substances. The study of their metabolic fate in animals has received much attention, even though there have been contradictory findings reported in the literature.

In a recent review by Willaman (1) on the biological effects of flavonoids, a summary of the data revealed that no less than thirty-three different types of physiological and biochemical activities have been reported for one or another of thirty different flavonoids. Physiological activity of a substance would suggest some sort of metabolism of that substance by the organism. Haley and Bassin (2) and Porter et al. (3) have indicated that rutin and related flavonoids gaining entrance to the body fluids and tissues are metabolized to a large extent. Previously, Garino (4) and Fukuda (5) had reported that rutin given orally to dogs or rabbits was found unchanged in the urine. The recent findings of Murray, Booth, De Eds, and Jones (6) substantiate the reports that oral administration of rutin or quercetin to rabbits results in appreciable absorption of these compounds from the gastrointestinal

tract, and that these compounds are metabolized. The resulting degradation products appear in the urine both in the free and conjugated forms.

Two-dimensional chromatograms of the ether extracts of rabbit urines before and after oral administration of rutin or quercetin to the animals according to Booth et al. (7) revealed the presence of two compounds in urine that are absent from control urines, and an increase in the amount of a normal constituent, m-hydroxyphenylacetic acid. One of the compounds found by these workers to be present in the urine from the flavonol-fed animals but absent from the urine of control animals is 3,4-dihydroxyphenylacetic acid. The second compound absent from control urine but present in the urine of the flavonol-fed animals was reported to be homovanillic acid.

These authors (8) suggested that the homovanillic acid arose from the 3,4-dihydroxyphenylacetic acid by methylation of the latter's meta-hydroxyl group. The possibility of such methylation by rabbits and by rats was investigated by the above chromatographic procedure. This method was applied to urines after oral administration of other 3,4-dihydroxyphenyl derivatives, namely protocatechuic acid and caffeic acid. Protocatechuic acid produced vanillic acid and caffeic acid produced ferulic acid.

Another point which was briefly investigated by Booth and coworkers (7) was the question as to whether quercetin degradation takes place in the intestinal tract or in the animal tissues after absorption from the gut. In order to eliminate possible effects due to digestive enzymes or intestinal bacteria, quercetin was administered by intraperitoneal injection. These authors state that unmistakable chromato-

graphic evidence of homovanillic acid was found in the urine, as was the case when quercetin was given orally. No explanation was presented for the absence of 3,4-dihydroxyphenylacetic acid and the failure to notice an increase of m-hydroxyphenylacetic acid in the urine of these animals. It was also stated that chromatographic analysis of the intestinal contents of rats receiving quercetin in the diet was negative in regard to the presence of quercetin metabolites found in the urine.

In an effort to discover the nature of the reactions involved in quercetin degradation, quercetin-2-C^{14} was synthesized at the University of Oklahoma by Mark Gutzke et al. (9), and quercetin-3-C^{14} was synthesized by T. H. Wei (10) for the animal studies. Also, randomly labeled quercetin was obtained for study by hydrolysis of rutin from buckwheat plants grown in a carbon dioxide-C^{14} atmosphere at Argonne National Laboratory. The radioactivity was distributed in several organs, and urine, feces, and exhaled carbon dioxide. This suggested certain possibilities with regard to the physiological role of quercetin.

Until the present study, all of the metabolic products of quercetin which have been identified appear to have originated from that portion of the quercetin molecule containing the catechol nucleus. The opposite side, or γ-pyrone portion, of the quercetin molecule had not been found.

The present study was, therefore, undertaken with both specifically labeled and randomly labeled quercetin in order to determine as many new, fundamental facts as possible concerning the fate of the entire quercetin molecule in the rat.

CHAPTER II

MATERIALS AND METHODS

The specifically labeled quercetin-2-C^{14} was synthesized by Gutzke (9) and the quercetin-3-C^{14} was synthesized by Wei (10) at the University of Oklahoma. The randomly labeled quercetin was obtained by the hydrolysis of rutin, which was kindly furnished by the Argonne National Laboratory. The rutin had been extracted from the leaves of buckwheat plants which had been grown in an atmosphere of radioactive carbon dioxide.

Hydrolysis of rutin. The hydrolysis of rutin was accomplished by refluxing for five hours, 151 mg. of this glycoside with 25 ml. of a solution which contained 2 ml. of concentrated sulfuric acid in 100 ml. of 20 % aqueous ethanol. After cooling, to insure the complete precipitation of the quercetin the reaction mixture was poured into a beaker containing 50 ml. of ice water. The yellow flocculant quercetin was recrystallized by dissolving it in ethanol and then adding sufficient water to effect complete precipitation of the flavonol. The yield was 96 % and the specific activity of the quercetin was 19.6 microcuries per millimole. The labeled quercetin was run on paper chromatograms in comparison with unlabeled synthetic quercetin, and with unlabeled quercetin obtained in pure form from buckwheat rutin, by hydrolysis. The labeled product

showed only one spot and gave the same R_f value as the two standards. The two solvent systems used in developing the paper chromatograms were n-butyl alcohol-acetic acid-water (6:1:2 v/v) and 60 % aqueous acetic acid. Radioautograms of the paper chromatograms which were developed in the above solvents showed only one radioactive spot, corresponding to quercetin.

Phenolic Acids Standard Chromatograms

In order to have comparison standards of the substituted phenolic acids which were anticipated as likely possibilities in the degradation of quercetin, it was necessary to synthesize a number of them which could not be easily purchased commercially. The syntheses were based on methods previously reported by others in the literature. Protocatechuic acid (3,4-dihydroxybenzoic acid) and homoprotocatechuic acid (3,4-dihydroxyphenylacetic acid) were obtained by the demethylation of the respective dimethoxy benzoic and phenylacetic acids. The demethylation was accomplished using 0.5 gm. of the methoxy acid and refluxing it for 18 hours with a mixture of 14 ml. hydrobromic acid (48 % HBr), 2 ml. hydriodic acid (sp. gr. 1.7), and 30 ml. glacial acetic acid. This demethylation mixture gave good results with a minimum of tar formation during the ether cleavage (11).

Vanillic acid was prepared by the oxidation of vanillin with silver oxide (12). Homovanillic acid was prepared from vanillin by converting the aldehyde to the alcohol, catalytically under pressure, using platinum as the catalyst. The alcohol was converted to the chloride, using thionyl chloride according to Berlin et al. (13). The resulting

vanillyl chloride was reacted with potassium cyanide, and the product was hydrolyzed with sodium hydroxide. The mixture was then acidified to give the homovanillic acid in relatively low yield. The product was recrystallized from cyclohexane to give pure homovanillic acid, m. p. 141 °C.

All the acids have been purified chromatographically, either on an adsorption column, or on paper, to give only one zone or spot with all the solvent systems used. The phenolic acids have been studied paper chromatographically, using the following solvent systems for ascending two-dimensional chromatograms: n-butyl alcohol-acetic acid-water (6:1:2 v/v) in the first direction and 15 % aqueous acetic acid in the second direction, and also chloroform-acetic acid-water (2:1:1 v/v) in the first direction, and 20 % potassium chloride in the second direction. For one-dimensional descending chromatography, n-butyl alcohol-acetic acid-water (6:1:2 v/v) and sec-butyl alcohol-water (3:1 v/v) were used. For spraying the developed chromatograms, use was made of diazotized sulfanilic acid prepared according to Bray et al. (14). The spray reagent, however, was made up by mixing the diazotized sulfanilic acid solution with an equal volume of 20 % aqueous sodium carbonate just before spraying. This was found to be a more sensitive procedure for the detection of very small quantities of the phenolic compounds. Bray et al. sprayed the papers first with the sulfanilic acid reagent and this was followed with 10 % aqueous sodium carbonate. The developed chromatograms were also sprayed with 1 % alcoholic aluminum chloride, methyl red, and a saturated aqueous solution of ammonium molybdate. From these, composite maps of the known, available phenolic acids were prepared. A small number of

these is shown in Figure 1. In Table I are shown the R_f values of some of these phenolic acids, and in Table II are shown the colors obtained after these acids were sprayed with the chromogenic reagents. The chromatographic behavior of a more complete list of phenolic acids has been reported by Bearden (15) and Kallianos (16).

The composite maps and R_f values were then used as reference standards for the identification of the radioactive spots found on chromatograms from the tissues, organs, or excreta of the rats fed the radioactive quercetin.

FIGURE 1

COMPOSITE MAP SHOWING THE RELATIVE POSITIONS OF SOME OF THE PHENOLIC ACIDS IN CHLOROFORM-ACETIC ACID-WATER AND 20 % POTASSIUM CHLORIDE

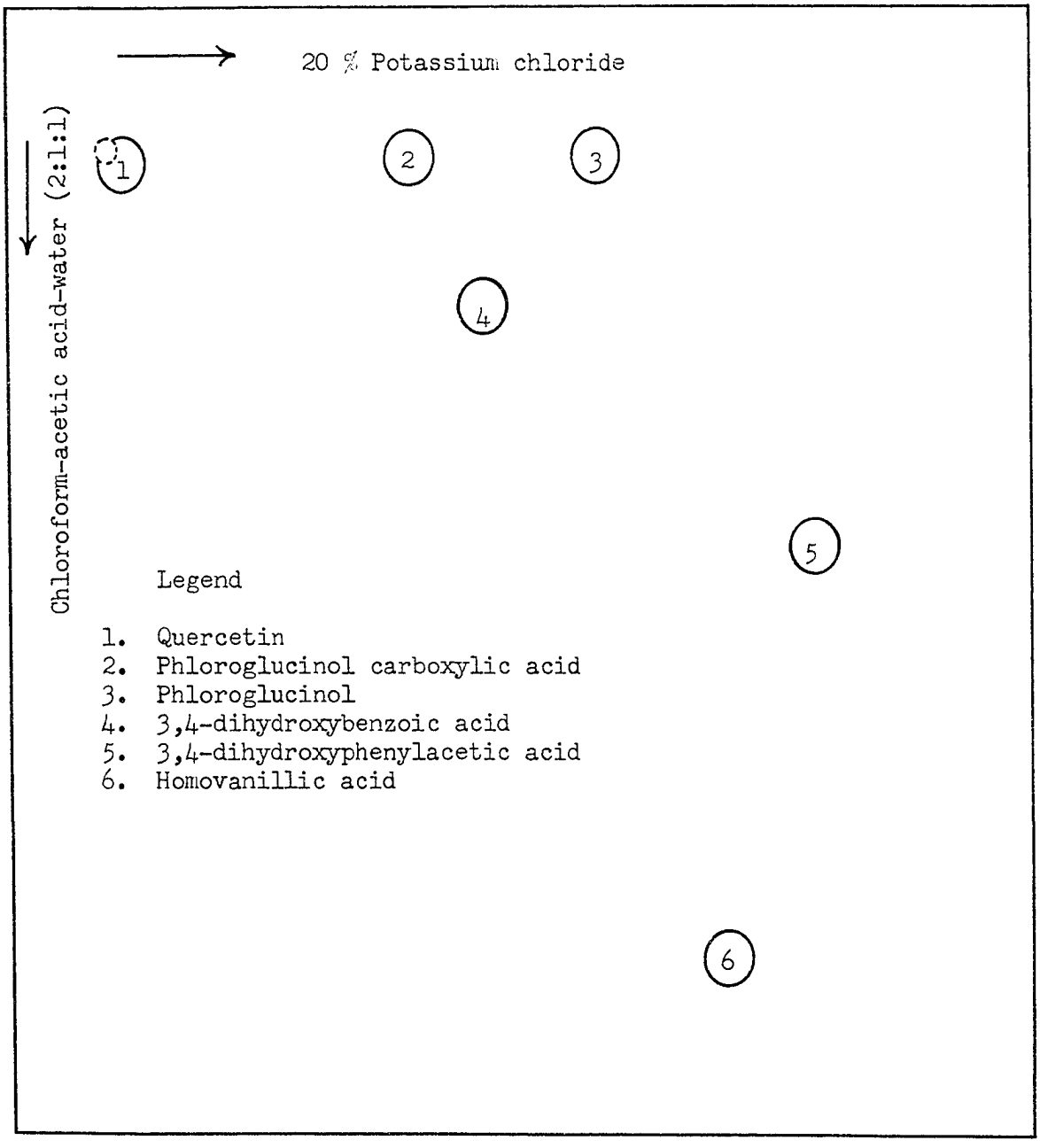

TABLE I

R_f VALUES OF SOME POLYPHENOLIC COMPOUNDS

compound	n-butyl alcohol acetic acid-water (6:1:2)	15 % acetic acid	chloroform-acetic acid-water (2:1:1)	20 % potassium chloride	75 % sec-butyl alcohol
3,4-dihydroxyphenylacetic acid	0.81		0.40	0.79	0.49
3,4-dihydroxybenzoic acid	0.78	0.64	0.16	0.42	0.63
Caffeic acid	0.80	0.53			
Phloroglucinol	0.76	0.70	0.01	0.55	0.87
Phloroglucinol carboxylic acid	0.55	0.68	0.01	0.34	0.50
Homovanillic acid	0.88	0.80	0.82	0.70	
Quercetin	0.78	0.07	0.00	0.00	

TABLE II

COLOR OF SOME POLYPHENOLIC COMPOUNDS TREATED WITH CHROMOGENIC SPRAYS

compound	without spray		methyl red	diazotized sulfanilic acid and 20 % sodium carbonate	ammonium molybdate
	ultraviolet	ultraviolet over ammonia			
3,4-dihydroxyphenylacetic acid	none	none	pink	pink	yellow
3,4-dihydroxybenzoic acid	none	dark blue	pink	pink	yellow
Caffeic acid	blue	blue	pink	brown	yellow
Phloroglucinol	none	blue	pink	yellow	none
Phloroglucinol carboxylic acid	none	none	pink	yellow	none
Homovanillic acid	none	none	pink	red	none
Quercetin*	yellow	dark yellow	none	yellow	yellow

*Yellow color under ultraviolet light after spray with 1 % alcoholic aluminum chloride.

CHAPTER III

ANIMAL STUDIES

The report by Schreiber and Elvehjem (17) that quercetin will prevent nasal excretion of a red (porphyrin?) pigment in young albino rats with a B vitamin deficiency under conditions of high humidity suggested the likelihood that investigations involving such animals should aid considerably in efforts to learn new facts about the fate and possible role of bioflavonoids in animal metabolism. Albino rats of the Holtzman strain were placed on a synthetic diet of Vitamin B Complex Test Diet, Riboflavin Deficient (Nutritional Biochemical Corp., Cleveland, O.) and these described herein as normal rats were placed on the above synthetic diet but with complete vitamin fortification. In both cases the animals were maintained on the synthetic diet for at least 30 days. After this period, the riboflavin deficient animals were examined for distinct deficiency symptoms like alopecia, vascularization of the cornea, loss in weight, and reddening of the fur, ears, nose, and claws.

The metabolism cage used for the following experiments was the all-glass type similar to the one described by Roth (18). Carbon dioxide-free air was drawn in the cage through one column of drierite and one column of soda lime, and this stream of air was constantly bubbled through an alkaline absorption column containing a solution of 0.5 N sodium

hydroxide, thereby trapping all exhaled carbon dioxide. This setup afforded several conveniences. The excreted feces and urine practically never mix and hence were available intact. This allows one to obtain a reasonably accurate count on either the feces or urine without any fear of contamination. The expired air is easily trapped, thereby making it possible to study any activity that it may have. The temperature inside the cage was maintained at about 26 °C.

The force feeding technique was employed, using a suspension of the radioactive quercetin in some kind of a liquid. The most satisfactory dispensing liquid was a 1 % aqueous solution of lecithin. This solution did not have any adverse physiological effects on the rats and did not alter the quercetin, as was found by chromatographic checks. Water was allowed ad libitum. The time each animal was allowed to remain in the metabolism cage was either 12 or 72 hours; the exact time is reported in the case history of each individual rat.

At the end of the experimental period, the animal was sacrificed, and the pertinent organs immediately removed, lyophilized and stored in the freezer. The exhaled carbon dioxide trapped in the sodium hydroxide solution was determined by titration with standard hydrochloric acid (19); the resulting solution was made up to a definite volume, and an aliquot was precipitated with 1 M barium chloride to give barium carbonate for counting purposes. Aliquots of these samples were counted in a gas-flow counter, using a carbon-14 standard and blanks. The radioactivity present in each organ was determined by counting a carefully weighed sample of the powdered organ. After a representative sample of each organ was weighed in a planchet, a few drops of ethanol were added with an eye

dropper. The alcohol helped wet the sample and made any loose fibers stick to the metal planchet. All samples were run in similar planchets, machined stainless steel cups, with a measured surface area of 4.7 cm^2. The organs were counted in a gas-flow counter D-47 manufactured by Nuclear Instrument and Chemical Corporation (Chicago). No corrections were made for self-absorption by the samples. The radioactivity was found to be widespread, but was relatively higher in the feces, urine, stomach, intestines, caecum and the gastrointestinal contents.

The organs that contained a considerable amount of radioactivity were extracted with ether several times over a period of about two days. The ether extracts of each organ were combined and then were reduced in volume in vacuo to almost complete dryness. The residue of all the organs as well as of the feces contained a certain amount of fat. The normal animals contained considerably more fat than the riboflavin deficient animals. Since the presence of the fat interfered with the chromatographic analysis of the samples, various procedures were attempted in order to separate the fat from the phenolic and radioactive components of the residue.

To the residue left after the evaporation of the solvent from the ether extracts of the organs were added about 10 ml. of n-pentane and 15 ml. of 50 % methanol. After shaking, the methanol layer was removed, and then the process was repeated. The pentane fraction which contained most of the fat was examined for radioactivity after the evaporation of the solvent, and was discarded whenever no activity was present. To the combined methanol extracts were added about 20 ml. water, and the resulting solution was extracted with ether. The ether extracts were dried over

sodium sulfate and reduced to a small volume *in vacuo*.

The extraction of the acidic substances from the residue left after evaporation of the solvent from the ether extracts of the organs was attempted also. The acidic compounds were dissolved by the addition of 20 ml. of 10 % sodium carbonate. The solution was filtered and extracted with ether to remove the non-acidic material. The sodium carbonate solution was cooled, neutralized and acidified to a pH of 2 with cold dilute hydrochloric acid. The resulting solution was extracted twice with ether. The combined ether extracts were dried over sodium sulfate and were reduced to a small volume.

However, when the presence of phloroglucinol carboxylic acid was suspected in some of the organs, a slightly different procedure was followed for extraction and defatting. This compound decarboxylates easily under the above conditions. The extraction of phenolic compounds from the organs was accomplished by using anhydrous ether. The evaporation of the ether extracts was performed in the same manner as above. The defatting of the residues was accomplished by adding small volumes of n-pentane, shaking and filtering off the pentane solution through a sintered glass funnel. The use of filter paper was avoided to prevent decarboxylation of the highly labile carboxyl group of phloroglucinol carboxylic acid. The process was repeated twice. The residual material in the flask was allowed to remain open to the air for a few minutes. A few drops of methanol were added to the sintered glass funnel that had previously been used to filter the pentane solution, in order to wash any phenolic compounds present in the funnel into the flask which contains the defatted residue.

The defatted residue was analyzed by one and two-dimensional chromatography in various solvents, using Whatman No. 1 filter paper. Solvent systems were selected so as to distinguish between the various phenolic compounds that were considered as possible degradation products of the quercetin that had been fed to the rat. These solvents included n-butyl alcohol-acetic acid-water (6:1:2 v/v), 15 % aqueous acetic acid, chloroform-acetic acid-water (2:1:1 v/v), 20 % potassium chloride, sec-butyl alcohol-water (3:1 v/v). The choice of solvents as well as of the chromogenic reagents which were used to spray the developed papers was altered slightly during the experiments in order to adjust to the best resolution and identification of the primary products found on the degradation of the flavonoid compound, and to eliminate a very large number of other possible degradation products that may be postulated theoretically.

The complete extraction with ether of the phenolic compounds, radioactive or otherwise, from the small intestines, large intestines, caecum, and the intestinal contents required additional treatment. These organs and contents were first autoclaved with 15 ml. of 0.1 N hydrochloric acid for 10 minutes, at 30 pounds per square inch of pressure and 115 °C. The flasks and contents were allowed to cool and then filtered. The residues were washed with small portions of ether, which after filtration were used to extract the appropriate aqueous solution. The ether extracts of each organ were combined, dried over sodium sulfate and reduced to a small volume <u>in vacuo</u>. The chromatograms of the organs and their contents after autoclaving generally were found to contain larger amounts of phenolic substances and larger amounts of radioactive phenolic

compounds than before autoclaving.

In the following pages, experimental details are described for each individual rat studied.

CHAPTER IV

INDIVIDUAL EXPERIMENTS CARRIED OUT WITH RATS

In the case of rat No. 1, the experiment was quite preliminary. The quercetin-2-C^{14} was dissolved in ethyl alcohol and force-fed. Five milligrams of the labeled quercetin was thus fed to the 148 gm. rat. The rat went into a coma and remained in that state throughout the period of the experiment. The rat was allowed to remain in the metabolism cage for four hours and then sacrificed. Pertinent organs were removed, homogenized, and counted. Most of the radioactivity was found in the gastric contents. The blood serum showed a significant count as well. Nothing else was done with the rat.

Rat No. 2 weighed 169 gm. and was allowed to remain in the metabolism cage for 16 hours. This time the 5 mg. of quercetin-2-C^{14} was suspended in glycerol and then given to the animal. It was noticed that the glycerol suspension of quercetin darkens on standing in the open air. Most of the radioactivity was found in the stomach and intestinal contents of the rat.

Rat No. 3 had been on the synthetic riboflavin deficient diet for two months. At the start of the experimental period this rat weighed 165 gm. and was fed 10 mg. of quercetin-2-C^{14}. The rat was placed in the metabolism cage and remained there for 72 hours. After the experi-

mental period the rat was sacrificed, dissected and various organs were examined for radioactivity. There was very little activity found in any of the organs. Most of the radioactivity was found in the feces and urine.

Rat No. 4 had been on the riboflavin deficient diet for 10 weeks. The rat weighed 209 gm. and was force-fed 5 mg. of quercetin-2-C^{14} in glycerol. After 72 hours, the animal was sacrificed and the pertinent organs were removed and lyophilized. Most of the radioactivity was found to be in the feces and urine. The feces of the rat were extracted with ether. The ether extracts were combined, reduced in volume and examined chromatographically. No compounds were identified in the ether extracts. The stomach, small intestines, large intestines, caecum and the intestinal contents were combined and extracted with ether. Due to the very low level of radioactivity found in the ether extracts of the combined intestinal organs, these ether extracts were combined with the ether extracts of a subsequent rat No. 6. The results are summarized in Table VII.

Rat No. 5 died immediately after the feeding of the quercetin-2-C^{14}.

Rat No. 6 had been kept on the riboflavin deficient diet for 12 weeks. The rat weighed 182 gm. and was fed 5 mg. of quercetin-2-C^{14}. The rat was sacrificed after 72 hours, and was then immediately dissected; pertinent organs were removed, lyophilized, and stored in a freezer. The urine and the feces of this rat contained the greatest amount of radioactivity. The combined ether extracts of the intestinal organs and contents of this rat and of rat No. 4 were examined chromatographically. R_f values in 15 % aqueous acetic acid, 60 % aqueous acetic acid, n-butyl

alcohol-acetic acid-water (6:1:2 v/v), chloroform-acetic acid-water (2:1:1), and 20 % potassium chloride, as well as the color obtained after spraying with diazotized sulfanilic acid, indicate the presence of 3,4-dihydroxybenzoic acid. Radioautograms of the various chromatograms revealed two radioactive areas. One area corresponded to the 3,4-dihydroxybenzoic acid. The other area did not show any color with the sulfanilic acid spray, but judging from its R_f value in the chloroform-acetic acid-water (2:1:1 v/v) system it may possibly be 3,4-dihydroxyphenylacetic acid.

The feces of Rat No. 6 have been found to contain considerably more radioactivity than possessed by the intestinal contents fraction. Paper chromatographic studies in various solvents and spraying with diazotized sulfanilic acid revealed the presence of 3,4-dihydroxybenzoic acid. A radioautogram of the chromatogram which had been developed two-dimensionally in chloroform-acetic acid-water and 20 % potassium chloride showed two radioactive areas. One area corresponded to 3,4-dihydroxybenzoic acid. The second area corresponded to homovanillic acid, judging from its R_f value in these solvents. The concentrations of these compounds are very low, and to obtain a darkening on the X-ray film the radioautogram was allowed to be exposed for 5 months. Due to this low concentration of the compounds it is not possible to produce visible colors after spraying with any chromogenic reagent. A summary of the results obtained is shown in Table VII.

Rat No. 7. This rat had been maintained on the synthetic diet with complete vitamin fortification. Preliminary experiments on the feeding of the animals had indicated that when quercetin was suspended in

glycerol, the entire suspension darkened on standing. In order to avoid any oxidation of the quercetin by the suspending medium, attempts were made to find a more desirable transfer medium. Tween 40 (polyoxyethelene sorbitan monopalmitate obtained from Atlas Powder Co.) did not darken after addition of quercetin and upon analyzing small samples of the suspension it was found that quercetin remained unchanged in this medium even after several days.

Rat No. 7 was fed 10 mg. of randomly labeled quercetin in 2 ml. of Tween 40. The syringe was washed with an additional milliliter of Tween 40 making a total of 3 ml. of this liquid that was fed to the animal. In the course of the 72 hour long experimental period the rat developed a rather severe case of diarrhea. Since the main purpose of our experiments has been the determination of the metabolic fate of quercetin, it is essential that absorption of this compound or any of its degradation products not be prevented by such physiological upsets as diarrhea. Nevertheless, in spite of the diarrhea, experimentation was undertaken. There was a relatively low activity present in the intestinal organs and their contents. These were, therefore, combined. They were autoclaved with 25 ml. of 0.1 N hydrochloric acid and extracted with ether. For rat No. 7, two main fractions were chromatographed. The combined intestinal organs and their contents comprised one fraction, and the feces comprised another fraction.

With the intestinal fraction, a major spot of radioactivity was found to correspond to protocatechuic acid (3,4-dihydroxybenzoic acid), both in R_f values in various solvents tested and in the color it produces with diazotized sulfanilic acid.

With the feces, two radioactive spots have been located on the paper chromatograms, both showing the same intensity of darkening on the X-ray film after a radioautogram was made. One spot corresponded to protocatechuic acid in papers run two-dimensionally in two different sets of solvents. One set of solvents was n-butyl alcohol-acetic acid-water in one direction and 15 % acetic acid in the second direction. A second set of solvents used was chloroform-acetic acid-water and 20 % potassium chloride. The second spot was found only on the radioautograms of the paper run in n-butyl alcohol-acetic acid-water and 15 % acetic acid. The appearance of this second spot in the latter systems and not on the radioautograms of the paper run in chloroform-acetic acid-water and 20 % potassium chloride possibly may be explained by the fact that whereas one radioautogram was developed after one month exposure, the other radioautogram was developed after three months' exposure. Evidently the second spot does not contain as much radioactivity as the first spot despite the approximately equal intensity of darkening. There was not enough material on the paper to give color with the diazotized sulfanilic acid spray, which is by far the most sensitive reagent used in this work.

The identity of this radioactive compound has not been determined.

The results of the determinations of radioactivity present in various organs of rat No. 7 are recorded in Table III. These counts have been corrected for background. A summary of the compounds found as degradation products of quercetin is given in Tables V and VI.

Rat No. 8. This rat had been maintained on the synthetic diet with complete vitamin fortification. Since the previous rat had diarrhea after the feeding of quercetin in Tween, it became desirable to find some

TABLE III

DISTRIBUTION OF RADIOACTIVITY IN VARIOUS ORGANS OF RATS NO. 7 AND NO. 8

Rat No. 7		Rat No. 8
Weight: 289 gm.		Weight: 309 gm.
Sacrificed after 72 hours		Sacrificed after 12 hours
Total counts/min. fed: 387,000		Total counts/min. fed: 193,500
Total amount fed: 10 mg. in Tween 40		Total amount fed: 5 mg. in lecithin
Organs	Total counts/minute	
	Rat No. 7	Rat No. 8
Stomach	1,230	67,000
Small intestines and contents	4,800	25,400
Large intestines and contents	9,300	71,420
Lungs	560	820
Liver	80	40
Kidney	620	210
Pancreas	36	80
Spleen	34	88
Heart	25	68
Testes	0	0
Fat	0	0
Feces	16,200	No fecal excretion
Blood	42	20

other medium to aid in the transfer of the quercetin to the animal. Lecithin was found to be quite good for this purpose. Therefore, the rat was force-fed 5 mg. of randomly labeled quercetin which had been suspended in 2 ml. of a 1 % aqueous solution of lecithin.

It had become evident that in order to be able to isolate any of the degradation products of quercetin in the organs of the rats, the experimental period should be shorter than 72 hours. The expired carbon dioxide which was collected from the previous rats was used as an indication of maximum metabolism of quercetin. It was found that the maximum amount of radioactivity in the carbon dioxide appeared at about 12 hours after the feeding of quercetin. The experimental time was, therefore, shortened to 12 hours. The stomach, small intestines, large intestines, caecum and the contents of each organ were extracted with ether and the resulting treated extracts were chromatographed on Whatman No. 1 paper. The small intestines and small intestinal contents as well as the large intestines and their contents were autoclaved. The ether extracts of the filtrate after autoclaving were reduced in volume and were combined with the ether extracts before autoclaving. The ether extracts of the organs were chromatographed. Two-dimensional chromatograms in chloroform-acetic acid-water and 20 % potassium chloride as well as in n-butyl alcohol-acetic acid-water and 15 % acetic acid and one-dimensional chromatograms in sec-butyl alcohol-water (3:1) indicate that a major fraction of radioactivity in the stomach, small intestines, and large intestines was due to 3,4-dihydroxybenzoic acid. This was identified by its R_f value in the various solvent systems used as well as by the colors that were produced after spraying with the diazotized sulfanilic acid reagent. In the

case of the stomach fraction the pink color with the diazotized sulfanilic acid spray was very prominent and was still visible months later.

To determine the nature of an unknown radioactive compound present in an extract, a small amount of the known compound suspected to be present in the extract was added. The mixture was chromatographed two-dimensionally and the position of the known compound was located by use of an appropriate spray. If the shape and size of the color developed spot corresponded exactly with the spot on the radioautogram of the same chromatographed paper it was assumed that the extract contained the known compound in radioactive form.

Additions of authentic samples of protocatechuic and homoprotocatechuic acids, one at a time, to the extracts, and chromatographing in n-butyl alcohol-acetic acid-water and 15 % acetic acid and in chloroform-acetic acid-water and 20 % potassium chloride supported the evidence presented for the presence of protocatechuic acid in the ether extracts of the organs. Also, at least in the stomach, it eliminated any doubt as to the absence of 3,4-dihydroxyphenylacetic acid. This compound had been reported by Murray (6) to be present in the urine of rats which had previously been fed quercetin. One other spot of about equal color intensity, in the stomach and large intestines and of a little less intensity in the small intestines is a yellow spot after spraying with diazotized sulfanilic acid. Its identity as phloroglucinol was confirmed on co-chromatography with authentic phloroglucinol, obtained from Ringwood Chemical Co., in sec-butyl alcohol-water (3:1). Still another spot, which sprayed yellow with diazotized sulfanilic acid, was found in the stomach fraction. It was of about equal radioactivity and color intensity as

phloroglucinol and checked on every chromatogram with authentic phloroglucinol carboxylic acid. The authentic phloroglucinol carboxylic acid was obtained from Morton Chemical Co. Attempts to isolate each of these compounds in sufficient amount and with high enough purity to determine their ultraviolet absorption spectra have not been successful thus far. The elution of the compounds from chromatographic papers introduces the interference of paper impurities (20). A summary of the results obtained is shown on Tables III, V, and VI.

Rat No. 9. This riboflavin deficient rat was fed randomly labeled quercetin. The animal had been maintained on a riboflavin deficient diet for 10 weeks. It was then fed 5 mg. of the randomly labeled quercetin in 2 ml. of lecithin solution. The animal was sacrificed after 12 hours, and the organs were lyophilized, and checked for radioactivity. The results are given in Table IV. Only the organs which contained a relatively high radioactivity were examined for the presence of degradation products of quercetin.

One and two-dimensional chromatograms in various solvents were run. Two-dimensional chromatograms in chloroform-acetic acid-water and 20 % potassium chloride revealed the presence, in the extracts from the stomach and large intestines, of protocatechuic acid after spraying with diazotized sulfanilic acid. A second chromatograph of the stomach extract in the same solvents indicated the presence of protocatechuic acid by giving a very faint brown spot, that was radioactive, at the location that known protocatechuic acid would be found. One and two-dimensional chromatograms of the extracts of the small intestines and their contents did not indicate the presence of any recognizable phenolic substance. A

brown spot, spraying with diazotized sulfanilic acid, appears on chromatograms of small intestines having an R_f value of 0.90 in the n-butyl alcohol-acetic acid-water system, with no movement in either 15 % acetic acid or 20 % potassium chloride, and streaking in chloroform-acetic acid-water. This spot contained most of the radioactivity extracted from the small intestines. Its identity has not yet been established.

The extracts of both stomach and large intestines have been shown chromatographically to contain phloroglucinol. The presence of phloroglucinol was indicated by the yellow spots obtained after spraying with diazotized sulfanilic acid on two-dimensional chromatograms in chloroform-acetic acid-water and 20 % potassium chloride and in n-butyl alcohol-acetic acid-water and 15 % acetic acid solvent systems. This phloroglucinol was eluted with anhydrous ether from two papers, one from each organ, stomach and large intestines, which had previously been run in chloroform-acetic acid-water and 20 % potassium chloride. The eluates were co-chromatographed with authentic phloroglucinol in sec-butyl alcohol-water (3:1). After development and spraying of the papers with diazotized sulfanilic acid, a yellow spot was obtained from the eluted fraction that corresponded exactly with the adjacent standard phloroglucinol. Both gave R_f values of 0.86 in this solvent. The unknown spot was shown to be radioactive.

The original ether extracts of the organs before autoclaving were spotted rather heavily on Whatman No. 1 paper and run in sec-butyl alcohol-water (3:1) along with several reference compounds. The reference compounds used were protocatechuic acid, homoprotocatechuic acid, phloroglucinol and phloroglucinol carboxylic acid. A pink spot after spraying

TABLE IV

RIBOFLAVIN DEFICIENT RATS FED RANDOMLY LABELED QUERCETIN

Rat No. 9	Rat No. 10
Weight: 169 grams	Weight: 180 grams
Sacrificed after 12 hours	Sacrificed after 12 hours
Total amount fed: 5 mg.	Total amount fed: 4 mg.
Total counts/min. fed: 193,000	Total counts/min. fed: 144,800

Organs	Total counts/minute	
	Rat No. 9	Rat No. 10
Stomach	1,780	6,000
Large Intestine, Contents, and Caecum	14,760	13,800
Small Intestine and Contents	4,680	11,000
Lungs	40	0
Liver	48	126
Heart	0	8
Spleen	20	10
Pancreas	0	0
Kidney	0	40
Harderian Glands	21	--
Feces	260	--

with diazotized sulfanilic acid and having an R_f value of 0.68 matched the standard 3,4-dihydroxybenzoic acid. The spot from the stomach showed about the same amount of radioactivity as the one from the large intestines. Another spot spraying yellow with diazotized sulfanilic acid and having an R_f value of 0.85 in this solvent corresponded fairly well with the standard phloroglucinol, although it was slightly less in R_f value. The lesser R_f value of the phloroglucinol in the extracts may possibly be explained on the basis that the extracts were spotted heavily on the paper, and the solvent front gets a little ahead before it gets a chance to penetrate the paper on the spot which contains a considerable amount of residual material. The radioactivity of the phloroglucinol on the papers was 22 counts/minute for the spot from the stomach extract and 30 counts/minute for the spot from the large intestinal extract. The total radioactivities spotted on the papers were 120 counts/ minute for the stomach extract and 150 counts/minute for the large intestinal extract. About one-half of this radioactivity moved with the solvent front. The identity of the compound or compounds in which this radioactivity is incorporated is unknown. In both extracts a brown spot was obtained after spraying with diazotized sulfanilic acid. It appears then that about one-half of the radioactivity extracted from the stomach, the large intestines, and the small intestines is some form of a phenolic compound as yet not identified.

A yellow spot at R_f 0.50 in sec-butyl alcohol-water (3:1) was obtained after spraying the chromatogram with diazotized sulfanilic acid. This spot from the stomach extract showed a radioactivity of about 25 % of the total spotted on the paper. This spot checked with phloroglucinol

carboxylic acid. No corresponding spot was observed in the large intestine extract. The presence of phloroglucinol carboxylic acid may not be conclusively proved from these data, since 3,4-dihydroxyphenylacetic acid has the same R_f value of 0.50 in this solvent. The yellow color cannot be taken as proof of the presence of phloroglucinol carboxylic acid and the absence of 3,4-dihydroxyphenylacetic acid, since at such a low concentration, the latter compound may not be as pink as it appears after spraying with the diazotized sulfanilic reagent on the standard sample in which it is present in a much larger concentration.

The presence of phloroglucinol carboxylic acid in the stomach extract was indicated by the use of two-dimensional chromatography. Satisfactory results were obtained with the use of chloroform-acetic acid-water in the first direction and 20 % potassium chloride in the second direction. The phloroglucinol carboxylic acid showed no apparent movement in the chloroform-acetic acid-water system, and an R_f value of 0.34 in the 20 % potassium chloride system. In the second direction, that is, in the 20 % potassium chloride, the phloroglucinol carboxylic acid found in the stomach extract was marked by some other phenolic substance present in this extract, which runs just behind the former compound. The identity of this substance is not known as yet but its presence has been confirmed by chromatography in other solvent systems and by radioautograms. This phenolic compound has thus far been encountered in extracts of the stomach of this rat and in the stomach of rat No. 8. In n-butyl alcohol-acetic acid-water, it has an R_f value of 0.44 and in 15 % acetic acid it has an R_f value of 0.78 when it is chromatographed two-dimensionally. A summary of the results obtained is shown in Tables V and VI.

TABLE V

CHROMATOGRAPHIC ANALYSIS OF THE DEGRADATION PRODUCTS OF RADIOACTIVE QUERCETIN IN THE EXTRACTS OF THE VARIOUS ORGANS

Fraction	Rat No.	BAW[a] (6:1:2)	15 % acetic acid	CAW[b] (2:1:1)	20 % KCl	75 % sec-BuOH[c]	Dev. Color[d]	Indicated Compound
Stomach	8	0.76	0.66	0.16	0.41	0.63	pink	3,4-dihydroxybenzoic acid
	8	0.74	0.68	0.00	0.58	0.86	yellow	phloroglucinol
	8		0.70	0.00	0.34	0.50	yellow	phloroglucinol carboxylic acid
	9	0.78	0.62	0.13	0.40	0.68	pink	3,4-dihydroxybenzoic acid
	9	0.74	0.68	0.00	0.55	0.86	yellow	phloroglucinol
	9	0.52	0.72	0.00	0.33	0.50	yellow	phloroglucinol carboxylic acid
	10	0.74	0.68	0.00	0.55	0.86	yellow	phloroglucinol
Large Intestines plus Caecum	8	0.78	0.69	0.16	0.40	0.68	pink	3,4-dihydroxybenzoic acid
	8	0.75	0.70	0.00	0.55	0.85	yellow	phloroglucinol
	9	0.78	0.66	0.16	0.41	0.66	pink	3,4-dihydroxybenzoic acid
	9	0.75	0.69	0.00	0.56	0.89	yellow	phloroglucinol
	10	0.75	0.69	0.00	0.56	0.89	yellow	phloroglucinol

TABLE V--Continued

Fraction	Rat No.	BAW[a] (6:1:2)	15 % acetic acid	CAW[b] (2:1:1)	20 % KCl	75 % sec-BuOH[c]	Dev. Color[d]	Indicated Compound
Small Intestines	8	0.74	0.62	0.16	0.40		pink	3,4-dihydroxybenzoic acid
Combined Intestinal tract and Content	7	0.73	0.61	0.14	0.40		pink	3,4-dihydroxybenzoic acid
Feces	7	0.76	0.69	0.16	0.44		pink	3,4-dihydroxybenzoic acid

[a]BAW: n-butyl alcohol-acetic acid-water

[b]CAW: chloroform-acetic acid-water

[c]75 % sec-BuOH: sec-butyl alcohol-water (3:1)

[d]Color developed with diazotized sulfanilic acid and 20 % sodium carbonate

TABLE VI

SUMMARY OF RESULTS

ANIMALS FED RANDOMLY LABELED QUERCETIN
List of compounds found to be radioactive in the various organs of rats

Rat	Stomach	Small Intestines	Large Intestine plus Caecum	Feces
Rat No. 7 Normal 72 hrs. (Diarrhea)	Not studied	3,4-dihydroxybenzoic acid (all intestines combined)		3,4-dihydroxy-benzoic acid unknown
Rat No. 8 Normal	3,4-dihydroxy-benzoic acid phloroglucinol phloroglucinol carboxylic acid	3,4-dihydroxybenzoic acid phloroglucinol	3,4-dihydroxybenzoic acid phloroglucinol	No fecal excretion
Rat No. 9 Riboflavin Deficient	3,4-dihydroxy-benzoic acid phloroglucinol phloroglucinol carboxylic acid	Unknown compound R_f 0.90 in BAW*	3,4-dihydroxybenzoic acid phloroglucinol	No fecal excretion
Rat No. 10 Riboflavin Deficient	3,4-dihydroxy-benzoic acid phloroglucinol phloroglucinol carboxylic acid	Unknown compound R_f 0.89 in BAW*	3,4-dihydroxybenzoic acid phloroglucinol	No fecal excretion

*BAW: n-butyl alcohol-acetic acid-water

Rat No. 10. This rat had been maintained on the riboflavin deficient diet for 11 weeks. The rat weighed 180 gm. and was fed 4 mg. of the randomly labeled quercetin. This rat was treated in a manner similar to the riboflavin deficient rat No. 9. Also, the results obtained were quite similar to the above mentioned rat. The distribution of radioactivity in the organs is shown on Table IV.

Since the livers of most rats studied thus far contained some radioactivity but not enough to warrant extraction of just one liver, the livers from three animals, rats Nos. 8, 9, and 10, were autoclaved with 15 ml. of 0.1 N hydrochloric acid and after filtration, the filtrate was extracted with ether. The ether extracts contained large amounts of fat and also residual material which was left after defatting with n-pentane. The residual material interfered very much with the accurate chromatographic analysis of these extracts. The radioactivity was distributed throughout the paper chromatograms which were run in n-butyl alcohol-acetic acid-water and 60 % acetic acid. It was impossible to cut any particular band from the papers for rechromatography in some other solvent systems. Streaking an extract on chromatographic paper, running it in some solvent that is appropriate for the satisfactory separation of the suspected substances, cutting the bands, and eluting directly onto another paper by trimming the end of the band and touching the narrowed end of the strip on a chromatogram which is then developed in a second solvent system, had proved to be a satisfactory procedure for rechromatographing purposes.

There was not enough material from rat No. 10 to run as many papers as had been run with rat No. 9. However, at least one chromatogram was run for each of the organs, stomach, small intestines, large intestines

TABLE VII

SUMMARY OF RESULTS OF THE DEGRADATION PRODUCTS OF QUERCETIN-2-C^{14} IN RATS

Rat	Stomach plus Small Intestines plus Large Intestines		Feces
Rat No. 4	3,4-dihydroxybenzoic acid 3,4-dihydroxyphenyl-acetic acid	(Stomach, small intestines and large intestines of Rats Nos. 4 and 6 combined)	No compounds identified
Rat No. 6			3,4-dihydroxy-benzoic acid Homovanillic acid

TABLE VIII

SUMMARY OF RESULTS OF THE DEGRADATION PRODUCTS OF QUERCETIN-3-C^{14} IN RATS

Rat	Stomach	Small Intestines	Large Intestines
Rat No. 11 Normal, fed 4 mg. of Quercetin -3-C^{14}	3,4-dihydroxy-benzoic acid* Phloroglucinol* Phloroglucinol carboxylic acid* unknown substance	No compounds identified	No compounds identified
Rat No. 12 Riboflavin deficient fed 4 mg. Quercetin -3-C^{14}	Phloroglucinol* Phloroglucinol carboxylic acid*	No compounds identified	Phloroglucinol*

*non-radioactive

and caecum, in the chloroform-acetic acid-water and 20 % potassium chloride and in the n-butyl alcohol-acetic acid-water and 15 % acetic acid solvent systems. Papers were streaked with the ether extracts of the large intestines and run in sec-butyl alcohol-water (3:1). By determining the radioactivity present at the position where phloroglucinol would normally appear and by spraying with diazotized sulfanilic acid enough phloroglucinol was found to be present to be identified.

The presence of 3,4-dihydroxybenzoic acid in the ether extracts of the small intestines and large intestines was indicated on paper chromatograms developed in the chloroform-acetic acid-water and 20 % potassium chloride system after spraying with diazotized sulfanilic acid.

Phloroglucinol was found to be present in the stomach and large intestinal extracts. The phloroglucinol carboxylic acid was found present in the ether extracts of the stomach and in the ether extracts of the large intestines before autoclaving.

The presence of phloroglucinol carboxylic acid had by this time been definitely established. Therefore, an attempt was made to determine whether the phloroglucinol carboxylic acid was decarboxylated in the intestinal tract of the rats to yield phloroglucinol or whether decarboxylation occurred during the treatment of the organs in the experimental procedure used. In order to use as mild conditions as possible, the intestines were extacted with anhydrous ether, before they were autoclaved. Paper chromatographic analysis of this extract indicated the presence of phloroglucinol carboxylic acid. A summary of the results is shown in Tables V and VI.

Rat No. 11. This rat was maintained on the synthetic diet with

complete vitamin fortification. The animal weighed 320 gm. and was force-fed 4 mg. of quercetin-3-C^{14}. The rat was allowed to remain in the metabolism cage for 12 hours. At the end of this period the rat was sacrificed and pertinent organs removed and lyophilized. Upon determination of the radioactivity of the organs it was found that only the stomach contained any appreciable amount of radioactivity. The specific activity of the quercetin-3-C^{14} which was used in this experiment was 0.0055 millicuries per mg. This gave about 2,400 counts per minute per mg. or a total of 9600 counts per minute fed to the rat. This was a very low level of activity, and attempts to isolate a radioactive substance after ether extraction of the stomach and chromatographic analysis of the ether extracts were not successful.

In order that some comparison be made with the previous rats the intestinal organs of this rat were extracted with ether. The ether extracts of the stomach, small intestines, and large intestines and caecum were analyzed by two-dimensional chromatography in chloroform-acetic acid-water and 20 % potassium chloride. After spraying the various chromatograms with diazotized sulfanilic acid, a yellow spot was obtained on the papers from the ether extracts of the stomach and the large intestines plus caecum. This spot corresponded to phloroglucinol. A yellow spot was obtained after spraying the chromatograms of the ether extracts of the stomach. This spot corresponded to phloroglucinol carboxylic acid. Another yellow spot was obtained after spraying with diazotized sulfanilic acid, which shows an R_f value of 0.30 in 20 % potassium chloride, no movement in chloroform-acetic acid-water, covers part of the phloroglucinol carboxylic acid, and was found to contain the majority of the radioactiv-

ity. More information will be needed in order to determine the identity of this substance. A summary of the results obtained is shown in Table VIII.

Rat No. 12. This rat had been maintained on a riboflavin deficient diet for 15 weeks. The animal appeared very sick after the force feeding of 4 mg. and remained immobile throughout the 12 hours. After the rat was sacrificed, the whole intestinal cavity was quite disrupted and bloody. Only the stomach of this rat was extracted with ether and the extracts were chromatographed in chloroform-acetic acid-water and 20 % potassium chloride. After spraying the developed chromatograms with diazotized sulfanilic acid, a yellow spot was obtained which corresponded to phloroglucinol. A summary of the results obtained is shown in Table VIII.

Rat No. 13. In order to eliminate possible effects due to digestive enzymes, quercetin was administered by intraperitoneal injection. This rat was given 4 mg. of randomly labeled quercetin.

Two-dimensional chromatographic analyses of the ether extracts of the intestines in chloroform-acetic acid-water and 20 % potassium chloride, in n-butyl alcohol-acetic acid-water and 15 % acetic acid were carried out. After development in these solvent systems the chromatograms were sprayed with diazotized sulfanilic acid. Only one phenolic spot which was radioactive was shown to be present in these chromatograms. Judging from its R_f value in n-butyl alcohol-acetic acid-water of 0.78 and the bright yellow color obtained after spraying with a 1 % alcoholic solution of aluminum chloride, the radioactive phenolic substance present was quercetin.

CHAPTER V

DEGRADATION OF QUERCETIN BY ENZYMES

From the experiments with the rats it was evident that quercetin is degraded in the stomach of the rat rather rapidly after the feeding. This was concluded from the fact that little quercetin is found in the ether extracts of the intestinal organs of the rat which had been fed radioactive quercetin. Compounds have also been extracted from the intestinal organs of the quercetin fed rat, which have been shown to be degradation products of quercetin.

Preliminary experiments with enzymes indicate that quercetin is degraded *in vitro* by some enzyme preparations of digestive origin.

The effect of some enzymes on quercetin. A glycine-sodium chloride-hydrochloric acid buffer solution was prepared by dissolving 0.1 moles of glycine in a liter of a 0.1 molar sodium chloride solution; to 25 ml. of the glycine-sodium chloride solution are added 25 ml. of 0.1 N hydrochloric acid. The pH of the buffer was 1.90. An acidic buffer was desirable for the high efficiency of the gastric enzymes which were used. To each of five test-tubes were added 10 ml. of the glycine-sodium chloride-hydrochloric acid buffer, and 1 mg. of quercetin. To four of the test-tubes containing the suspension of quercetin in the buffer solution were added about 1 mg. of the following enzymes:

Pepsin (2X crystalline) obtained from Nutritional Biochemicals Corp.

Rennin (crystalline) obtained from Nutritional Biochemicals Corp.

Pepsin, obtained from Armour Laboratories

Gastric Mucin obtained from Nutritional Biochemicals Corp.

A tube containing a suspension of quercetin in the buffer was used as a control.

The tubes were incubated at 42 °C for 72 hours. At the end of this time the solutions were extracted with ether. The reduced ether extracts from each mixture were chromatographed in chloroform-acetic acid-water and 20 % potassium chloride. After development the chromatograms were sprayed with diazotized sulfanilic acid. From the ether extracts of the recrystallized pepsin, gastric mucin, and rennin a yellow spot was obtained after spraying with the sulfanilic acid reagent, which corresponded to phloroglucinol. A yellow spot after spraying with diazotized sulfanilic acid was present on the chromatogram of the ether extract of gastric mucin, which corresponded to phloroglucinol carboxylic acid. The catechol part of the quercetin molecule was not shown to be present in the ether extracts of the solutions in these preliminary experiments.

CHAPTER VI

SUMMARY

Two-dimensional chromatograms of the ether extracts of the lyophilized gastrointestinal organs of rats which had been force-fed radioactive quercetin indicate that this flavonol is degraded quite rapidly in the stomach of the rat. The paper chromatograms were sprayed with diazotized sulfanilic acid to detect phenolic compounds. Administration of radioactive quercetin to rats which had been maintained on a normal synthetic diet with complete vitamin fortification and to other rats which had been maintained on a synthetic diet devoid of riboflavin resulted in the appearance of five radioactive spots of phenolic character in the ether extracts of the gastrointestinal organs and of the feces.

One of the radioactive spots which was present on the chromatograms of the gastrointestinal organs of every rat studied was 3,4-dihydroxybenzoic acid, according to chromatographic evidence. This was confirmed on rats fed randomly labeled quercetin and on rats fed quercetin-2-C^{14}. Phloroglucinol and phloroglucinol carboxylic acid have been discovered in the ether extracts of the stomach of the rat. Until the present study, the metabolic products of quercetin which have been identified appear to have originated from that portion of the quercetin molecule containing the catechol nucleus. The γ-pyrone portion of the quercetin

molecule had not been found.

Two other radioactive phenolic compounds which were found have been identified tentatively as 3,4-dihydroxyphenylacetic acid and homovanillic acid. The former compound was found in the combined ether extracts of the gastrointestinal organs of rats fed quercetin-2-C^{14}. The spot corresponding to the latter compound was present in the ether extracts of the feces. The exact position, size, and shape of each radioactive spot on the paper were determined by making radioautograms of the two-dimensional chromatograms.

Preliminary experiments have been undertaken on the degradation of quercetin by various enzyme preparations. Thus far both phloroglucinol and phloroglucinol carboxylic acid have been identified by two-dimensional chromatography of the products obtained by the degradation of quercetin by gastric mucin.

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