THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE SYNTHESIS OF QUERCETIN-2-C^{14}

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

MARK ELIAS GUTZKE

Norman, Oklahoma

1956

THE SYNTHESIS OF QUERCETIN-2-C^{14}

APPROVED BY

Simon H. Wender

Bernard O. Huston

J. C. Colbert

Kenneth T. Conrad

J. B. Clark

DISSERTATION COMMITTEE

ACKNOWLEDGMENT

The author wishes to express his deep appreciation to Dr. Simon H. Wender for suggesting this problem, for providing a fellowship from funds made available to him and for his counsel and encouragement during the course of this research. He would also like to express his gratitude to the Research Corporation for the F. G. Cottrell grant which provided the funds for this investigation.

The author wishes to thank Charles Nystrom, Reagan Bradford, Chao-Hwa Yang, Edwin Murphy and other fellow graduate students for their assistance and fellowship during this research. Finally, he wishes to thank the members of the Chemistry Department for their instruction and fellowship during his stay at this university.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The use of radioisotopes as tracers in biological studies was begun in 1923 by Hevesy,[1] who used radioactive lead to investigate the uptake and distribution of this element in plants. Since that time, and especially since World War II, a large number of isotopes, both stable and radioactive, both natural and artifical, have become available and have been used as tracers in almost all fields of science.

The specific advantages of isotopically labeled elements and compounds have been reviewed by several workers.[2-6] Kamen, in his review[7] oversimplifies the tasks when he points out that it is possible, by means of isotopic labeling, "----to distinguish and trace any molecule or atomic grouping the behavior of which is of interest in connection with biological function."

One of the most important isotopes that is used in the biological fields today is C^{14}, the long-lived radioactive carbon isotope. Two of the inherent advantages of this isotope have to do with its long half-life and with its

comparatively soft radiations. The half-life of C^{14} is approximately 5,600 years[8] and therefore requires no decay corrections in assay procedures. The radiations from C^{14} have an average range in air of approximately 4-6 cms and are in the form of a simple negative beta spectrum with a maximum energy of approximately 0.15 million-electron-volts.[9] These soft radiations keep the radiation damage of living tissue at a very low level, while in addition, they give the experimenter a very useful method of assay.

There are two major, general methods of synthesizing labeled compounds. One involves the "laborious efforts" of the organic chemist, and the other involves the synthetic ability of living tissue. This latter method, biosynthesis, is primarily used when the synthesis of the compound sought is extremely difficult or impossible. For example, it has been used to obtain such complex compounds as proteins[10] and lipids.[11] These biosynthesized compounds are usually uniformly labeled. In some cases, however, they are very difficult to purify to a satisfactory degree.

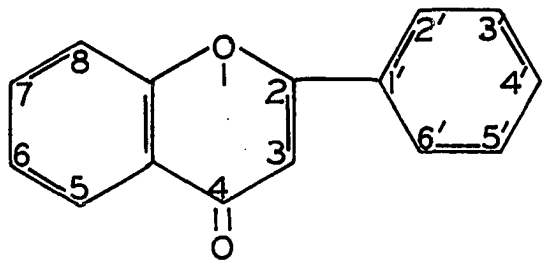
The laboratory procedures, where possible to be utilized, result in substances which have several advantages over compounds produced by biosynthetic methods. The ability to obtain specifically labeled compounds gives the scientist a chance to follow the specific atom or grouping throughout a metabolic scheme. In addition, it is possible to obtain compounds which have the labeled atom strategically placed

so that it is not likely to become part of a metabolic pool. The laboratory synthesis, as against the biological method, also affords a much greater control over the dilution of the isotope, since corresponding non-labeled reactants may be introduced, where needed, in known quantities. These controls over dilution are limited, of course, by the activity of the starting material and the yields of the various reaction steps.

Since this thesis reports on the laboratory synthesis of a specifically labeled flavonoid, a brief discussion of flavonoids and their status in biochemistry is given below.

The field of flavonoids, a group of naturally occurring substances related to the 2-phenyl-γ-benzopyrone structure, (Figure 1) was strongly associated twenty years ago with biological activity in animals, when certain plant

Figure 1.



Numbering system of the 2-phenyl-γ-benzopyrone structure.

extracts, such as those from lemon and paprika, and shown to contain flavonoid compounds, were reported to be beneficial in the treatment of adverse capillary conditions in man.[12] Although there are conflicting reports,[13-15] this work has been confirmed and expanded so as to suggest that the flavonoids may play a role in the metabolic fight against a number of other pathological conditions and diseases.

By citing a few examples from the literature, one may visualize the great and varied amount of work that has been done concerning flavonoids as theraputic agents. Only a brief summary of the flavonoid field will be given here, since a number of reviews have already been written concerning the status of flavonoids in relation to their so-called "vitamin P" activity and other biological effects and with respect to their chemistry.[16-20]

In 1943, Sevin announced that rutin, a flavonoid glycoside, would increase the capillary resistance of normal guinea pigs as determined by a suction method[21] and in 1948, flavonoid therapy was reported to be of value in experimentally induced frostbite in rabbits.[22] In relation to excessive irradiation from x-rays or radioactive elements, Rekers and Field[23] found that rutin, administered three times a day in 50 mgm. quantities to mice, assisted in controlling the syndrome, and it reduced the mortality rate in mice from sixty-two to twelve percent. Eddy and Sokoloff[24] have reported on the results of an analysis of 300 case

histories compiled by 22 radiotherapists. These results indicated that citrus flavonoid "reduces radiation erythema up to 60-90% and increases considerably the tolerance to deep radiation".

Schoenkerman and Justice[25] suggest that there is a sufficient increase in the relief of allergy symptoms when flavonoids are combined with antihistamines, to warrant further investigation, while Boines[26] has suggested the use of flavonoids as an adjunct in the over-all clinical management of poliomyelitis patients. A recent popular article reports the use of flavonoids in therapy of the common cold.[27]

Finally, one can mention the work by Griffith, et.al.[28] concerning old age. They give clinical data which they conclude show that flavonoids reduce the incidence of mortality associated with capillary fault. By using statistics on the clinical data and mortality, they claim that this treatment "prevents eighty-eight out of every one hundred deaths due to apoplexy" and "a sixty-five percent reduction in deaths due to coronary occlusion".

That the flavonoids do play some role in animal metabolism still is considered a possibility and certain investigators support the belief that they may play an important role. However, no definite metabolic function has been assigned to them. With the use of radioactive tracers, research workers in the field hope that some data can be ob-

tained concerning the possible path flavonoids take in metabolism and hope to get information as to their possible role.

With the knowledge that flavonoids occur in many vegetable foodstuffs and that they have been associated with nutritional requirements, it is significant to note that with three possible exceptions, all the naturally occurring flavonoids found have been reported in the plant kingdom. One exception is the report by Thomson,[29] which showed that a quercetin-like flavonoid is present in the wings of the butterfly. He also reports, however, that this might be of plant origin. The second exception is the report by Eddy and Sokoloff,[30] who claimed to have obtained evidence for the presence of two flavonoids in the adrenal glands of cows. By using the Wilson borocitric reaction[31] on adrenal gland extracts they were able to produce spectophotometric curves which had absorption peaks similar to those obtained with flavonoids which had been treated with the borocitric reagent. Similar spectophotometric curves have been obtained, by other workers but further work on attempted isolations has not produced any definite flavonoid compounds.[32,33]

The final exception, and probably the most noteworthy, is a report by Szent-Györgyi concerning his isolation of a yellow solid from the thymus gland.[34] This substance shows some reactions which characterize flavonoids, such as its reaction in alkali, its reaction with poly-

valent ions like Zn^{++} and Al^{+++}, and its light absorption spectrum. Up to this time, however, no report has been published concerning its identity.

These reports and others concerning the physiological action of flavonoids have prompted other investigators to increase their studies concerning the chemical nature of the flavonoids and their occurrence in nature. Since most of the flavonoids and the "vitamin P" preparations are obtained from plant sources, the problem of purity has been a factor in metabolic studies. In addition to this, most, if not all, crude flavonoids that have been used in these investigations contain several related flavonoids, and assigning a role to any one of them would be extremely difficult. Another problem is that a large number of foodstuffs contain these compounds and the problem of excluding these flavonoids from the diet of animals increases the difficulty of solving the cause and effect relationship of a specific flavonoid in metabolism. In 1949, Wender and coworkers[35-37] reported on methods for the isolation, separation, purification and characterization of flavonoids and related compounds utilizing paper and column chromatography. It can be readily seen that such methods will be an important tool in determining the fate of a labeled flavonoid compound in a biological system.

With this information in mind, the present investigation was undertaken as part of a large preliminary survey

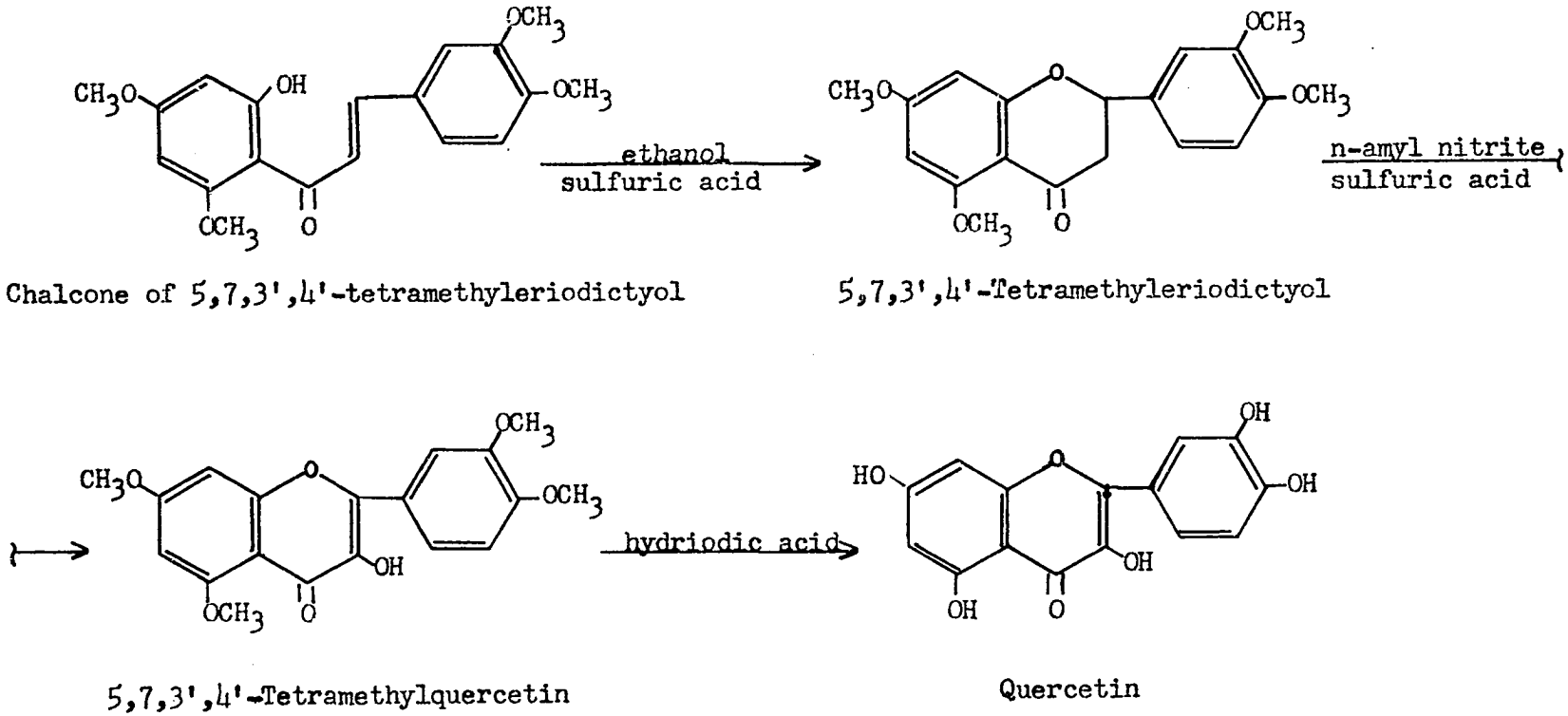
by these laboratories and concerned with the eventual elucidation of the fate of flavonoids in biological systems. It is specifically concerned with the synthesis of a flavonoid which contains a tagged carbon atom in a pre-designed position.

The flavonoid chosen for this work was quercetin, 2-(3,4-dihydroxyphenyl-) 3,5,7-trihydroxybenzopyrone. The reasons for this choice were twofold. Quercetin is one of the more widely distributed flavonoids in the plant kingdom and, in addition, it has been reported to possess a very high therapeutic activity.[37]

Quercetin was first synthesized by Kostanecki, et.al.[39] in 1904 using as a starting material the chalcone of 5,7,3', 4'-tetramethyleriodictyol and proceeding through a series of reactions shown in Figure 2. This chalcone had been previously prepared by the same author from veratraldehyde and the dimethyl ether or phloroacetophenone.[40] Reactions similar to those used in Kostanecki's work and modified by D. W. Fox[41] were followed in this work.

To conserve the activity in labeled syntheses, a number of adaptations and modifications were found and utilized in the present study. In radioactive syntheses, it is advantageous to have reactions where the labeled reactant is most highly utilized. Methods for the recovery of unreacted labeled compounds and by-products, where applicable, are also advantageous. This affords the re-

Figure 2.



Chalcone of 5,7,3',4'-tetramethyleriodictyol

5,7,3',4'-Tetramethyleriodictyol

5,7,3',4'-Tetramethylquercetin

Quercetin

Kostanecki's synthesis of quercetin.

searcher with some labeled intermediates for other syntheses or metabolic studies or increases the amount of product in subsequent runs. The reactions must also be adapted so as to be carried out on a small scale.

Since the starting material for these syntheses was to be potassium cyanide-C^{14}, it was necessary to begin further back and prepare one of the starting materials used by Kostanecki. In looking at the structure of quercetin (Figure 2) it was decided to have the labeled carbon atom in the number two position. Since this position is part of the heterocyclic ring, it is hoped that exchange reactions in a biological system will not readily occur. If the molecule is metabolized by the system, there should also be a good chance that the labeled atom will follow a fragment that might stay intact and that will be readily identified and followed further.

The purpose of this thesis research was, therefore, to devise a series of syntheses by which commercial potassium cyanide-C^{14} could be converted into veratraldehyde containing the tagged carbon atom in the carbonyl position and to convert this product, by a series of reactions on a small scale, and in reasonably good yield, into pure quercetin-2-C^{14} as the final product.

CHAPTER II

EXPERIMENTAL

Preliminary Investigations on the Synthesis of Labeled Quercetin

Several synthetic routes, concerned with the synthesis of quercetin, were undertaken and are described below. It was thus necessary to prepare a number of intermediates, both for use in the syntheses with labeled material and for use in the preliminary studies. The preparation of these intermediates is also described below.

Preparation of veratrole (o-dimethoxybenzene)

Since veratrole was used as a starting material in a number of syntheses of veratraldehyde, this reaction was carried out a number of times and in rather large quantities. Veratrole was prepared according to the method of Barger and Silberschmidt.[42]

In the hood, a three-necked flask was charged with 124 gm. (1 mole) of freshly distilled guaiacol (o-methoxyphenol) and then fitted with a mechanical stirrer and two dropping funnels. The flask was heated with a water bath of 60°C. A solution of 86 gm. (1.5 mole) of potassium

hydroxide in 100 ml. of water was added dropwise from a funnel at the rate of about two drops per second, while the entire solution was being stirred. After about twenty seconds, the addition of 118 ml (158 gm; 1.25 mole) of dimethyl sulfate was started dropwise, at the same rate, from the second funnel. After the addition of about 10 ml of the potassium hydroxide solution, the external heating was stopped. The reaction was kept a reddish-brown in color, indicating an excess of base as opposed to a green color obtained when the dimethyl sulfate was added too rapidly. With this indicator, it was possible to keep the reaction slightly basic at all times. When about three-fourths of the reagents had been added, a second phase appeared. After the addition was complete, the mixture was allowed to cool and the resulting veratrole was extracted with ethyl ether. This solution was dried over anhydrous magnesium sulfate. After carefully evaporating the ether, the veratrole was distilled under reduced pressure. The boiling point was 50-53°C under a pressure of about one mm of mercury. The product was obtained as a pale yellow oil and weighed 110 gm (80% of theoretical). It gave no color with ferric chloride.

In a similar experiment, catechol (o-dihydroxybenzene) gave veratrole in a seventy-five percent yield. Repeated attempts to crystallize veratrole were not successful and the product was used as an oil in all subsequent reactions.

Veratraldehyde by the Gattermann synthesis

The Gattermann synthesis is a method by which hydroxy aldehydes may be obtained from zinc cyanide and the corresponding hydroxy compound in one reaction. For this reason, studies on the preparation of veratraldehyde, by this method, were undertaken as a possible means of introducing, in reasonably good yield, a labeled carbon atom in the carbonyl position of the aldehyde.

Preparation of zinc cyanide. A solution containing 13.6 gm (0.1 mole) of c. p. zinc chloride dissolved in a minimum amount of 50% ethanol was added immediately to a solution containing 13 gm (0.2 mole) of potassium cyanide (96-98% pure) dissolved in 17 ml of water. A white precipitate of zinc cyanide formed at once and was filtered with suction. The precipitate was washed with 95% ethanol and then with ethyl ether and finally dried in a desiccator. The yield was 10 gm or 85% of theoretical.

By titrating the cyanide present with a standard silver nitrate solution, the zinc cyanide was found to be about 90% pure. The impurities probably are zinc chloride and basic zinc cyanide, which are said[43] not to interfere with the Gattermann reaction. It has also been shown[44] that purified zinc cyanide does not work as well in this reaction as the more impure zinc cyanide.

The yields and purity of zinc cyanide from the above reaction varied rather widely. The yields of pure zinc

cyanide varied from 60-80%. This was contaminated from 5-50% with impurities. When technical sodium cyanide was used, it was purified by adding a solution of magnesium chloride to precipitate the hydroxyl and carbonate ions which contaminated the sodium cyanide to the extent of 7-8%. Otherwise the zinc cyanide turned black on standing.

Preparation of the aldehyde. A dry 250 ml, three-necked flask was equipped with a gas inlet tube, a mechanical stirrer, and a condenser fitted with a drying tube. This flask was charged with 14 gm (0.12 mole) of zinc cyanide (90% pure) as prepared above, 10 gm (0.07 mole) of veratrole and 45 ml of dry benzene. After cooling to 0°C in an ice-salt bath, the stirrer was started and hydrogen chloride was bubbled in at a rapid rate.

The hydrogen chloride was prepared by carefully adding concentrated sulfuric acid, drop by drop, into a paste of sodium chloride and concentrated hydrochloric acid. The gas was then dried by bubbling it through concentrated sulfuric acid.

After thirty minutes the reaction was interrupted, and 15 gm of anhydrous aluminum chloride was added. The temperature was raised to 45°C and hydrogen chloride was passed into the reaction vessel for another two hours at a rather slow rate, one bubble per second. After cooling, the flask was stoppered and placed in the ice box overnight.

The aldimine hydrochloride which appeared as a yellow

crystalline solid was filtered and then decomposed by refluxing with 100 ml of a 10% hydrochloric acid solution for thirty minutes. By extracting this solution with ethyl ether, the aldehyde was obtained as an oil and weighed 5.7 gm. This is a 50% yield based on the veratrole but only a 14% yield based on the cyanide.

A 2,4-dinitrophenylhydrazone of the veratraldehyde was prepared and had a melting point of 258°C. The literature reports 265°C.[45]

The Gatterman reaction was also carried out with the molar ratio of the reactants being varied down to 1 mole of veratrole and 1.5 moles of cyanide. The yields of the aldehyde, based on the cyanide, were not improved.

During the entire course of the reaction, the exhaust gases were led from the condenser, through the drying tube and through a U-tube containing distilled water, into an ethanolic solution of zinc chloride. Since the cyanide was to contain the labeled carbon atom, it was hoped that the excess cyanide could be trapped as zinc cyanide and recycled through this reaction. In this manner, however, only a 7% recovery of the initial cyanide was obtained. Moreover, the resulting zinc cyanide was assayed with silver nitrate to be 70% pure and gave no aldehyde when recycled.

Due to the low conversion of zinc cyanide to veratraldehyde, the Gattermann synthesis was not used in the synthesis of labeled quercetin. Methods for the preparation

of veratronitrile were then studied with the thought that this nitrile could be converted to veratraldehyde.

Veratronitrile by the diazotization reaction

The preparation of veratronitrile by the action of cuprous cyanide on the diazonium salt of 4-aminoveratrole was undertaken. In order to obtain the aminoveratrole it was necessary to nitrate veratrole and reduce the product, 4-nitroveratrole. The nitrile resulting from this series of reactions would then be reduced to the corresponding aldehyde, which would then be converted into quercetin. The starting materials for this series of reactions were veratrole and potassium cyanide.

Preparation of 4-nitroveratrole. The method of Clark[46] was used to prepare the nitroveratrole. A solution of 20 gm (0.145 mole) of veratrole in 20 ml of glacial acetic acid was added dropwise into a stirred solution of 25 ml of concentrated nitric acid in 50 ml of distilled water. This solution was kept at 0°C with an ice-salt bath. After the addition was complete, the stirring was continued for one hour at 0°C and for a second hour at room temperature. The crystals which formed were filtered with suction and washed with cold water. After recrystallizing from dilute methanol the nitroveratrole was dried in a desiccator. The yield was 25 gm or 94%. The melting point was 98°C. Clark gives 96°C.[46]

Preparation of 4-aminoveratrole. A solution of 25 gm (0.14 mole) of 4-nitroveratrole in 100 ml of 95% ethanol was reduced at room temperature in a low pressure hydrogenation apparatus. This apparatus was equipped with a shaking device for the reaction vessel. The catalyst was 0.3 gm of palladium black. A fifty pound hydrogen pressure was used and the reaction vessel was shaken during the reaction. At the end of one hour the reaction was stopped. The alcohol solution was then reduced in volume by evaporation, to about 40 ml and the aminoveratrole precipitated by adding n-pentane. After recrystallizing from ethyl ether, the product was obtained as white plates and weighed 18 gm (85%). The melting point was 85°C. Clark[46] gives 81°C.

When the catalytic reduction was carried out at 100°C using Raney nickel as the catalyst, a 35% yield was obtained. A 52% yield was obtained when the reduction was carried out using tin and concentrated hydrochloric acid.

Preparation of veratronitrile. A solution containing 15 gm (0.1 mole) of 4-aminoveratrole and 7 ml (13 gm; 0.13 mole) of concentrated sulfuric acid in 100 ml of distilled water was prepared and cooled in an ice-salt bath. This solution was kept at 0°C and stirred by hand while 7 gm (0.1 mole) of sodium nitrite in 15 ml of water was added dropwise from a dropping funnel. The stirring was continued for fifteen minutes and then an additional 2 ml of concentrated sulfuric acid was added.

A cuprous cyanide solution was also prepared by dissolving 13.5 gm (0.15 mole) of cuprous cyanide in a solution of 10 gm (0.15 mole) of potassium cyanide in 20 ml of water. The preparation of the cuprous cyanide will be described in synthesis of labeled cuprous cyanide.

The cuprous cyanide solution was warmed to about 60°C and the cold diazonium salt solution was added in small portions. After each addition, the solution was stirred vigorously, and during the entire reaction the temperature was maintained at about 60°C. After the addition was complete, the solution was heated at 60°C for an additional fifteen minutes. The solution was then allowed to cool and was extracted with toluene. After removing the toluene under reduced pressure, an oil remained. Crystals appeared on standing.

A chromatographic column was prepared by adding a slurry of magnesol in ether to a chromatographic tube and packing the adsorbant under 8 pounds of air pressure. Dry ethyl ether was used as the solvent. This column, 1.8 x 20 cm, was washed with ether. The crude veratronitrile obtained above was dissolved in 25 ml of anhydrous ether and passed through the column. The nitrile came through the column with the solvent front. The column was then washed with ether.

The eluate containing the bulk of the nitrile and the washings were combined. The ether was then removed under re-

duced pressure. The nitrile was obtained as white crystals and weighed 7.7 gm. This is a 45% yield based on the aminoveratrole, but only a 16% yield based on the cyanide.

The preparation of veratronitrile, by the action of cuprous cyanide on the diazonium salt of 4-aminoveratrole, was not used with the labeled material since another method was found that would give a higher conversion of the cyanide ion into veratronitrile. The later method involved a reaction in which cuprous cyanide was reacted with iodoveratrole to produce veratronitrile in reasonably good yields. The preparation of 4-iodoveratrole was, therefore, necessary.

Preparation of 4-iodoveratrole. A solution of 28 gm (0.2 mole) of veratrole in 75 ml of 95% ethanol was heated to 60°C and, while stirring, was treated with 50 gm of iodine and 30 gm of mercuric oxide. The iodine, in 5 gm portions, and the mercuric oxide, in 3 gm portions, were added alternately over a period of one hour. Each time the iodine was added, the color of the solution turned to a deep purple. This purple color was allowed to disappear before another quantity of iodine was added. After the addition was complete, the solution was filtered and the alcohol was distilled from the filtrate. The residue, a dark red oil, was dissolved in ethyl ether and washed with solutions of sodium thiosulfate and sodium hydroxide, and finally with water. These washings took out most of the color, and left a pale yellow solution.

After drying over anhydrous magnesium sulfate, the ether was evaporated and the residue distilled under reduced pressure. This gave 25 gm of a heavy pale yellow oil. The boiling point was 80-85°C under 1 mm pressure of mercury. A portion of this oil crystallized after standing about one month in the ice box. Subsequent oils were seeded with these crystals.

The reaction of this product, 4-iodoveratrole, with cuprous cyanide-C^{14} is described in later paragraphs. Once this method was available for preparing veratronitrile, studies were undertaken to find suitable methods of converting it to veratraldehyde.

Attempted preparation of veratraldehyde by the Stephen's reaction

The Stephen's reaction is a method by which aromatic nitriles may be reduced to the corresponding aldehydes. The catalyst used in this reaction was anhydrous stannous chloride. A method for preparing it with reproducible results is given below.

Preparation of anhydrous stannous chloride.[47] Anhydrous stannous chloride was prepared by adding 1 mole of crystalline c.p. stannous chloride dihydrate to 2 moles of freshly distilled acetic anhydride, with stirring. The dehydration caused the solution to boil. After ninety minutes, the anhydrous stannous chloride was filtered with suction, washed with two 50 ml portions of ethyl ether, and

dried in a vacuum desiccator. Yields of over 95% were obtained.

The effectiveness of this catalyst and of the following procedure was found to be satisfactory by first preparing p-tolualdehyde from its corresponding nitrile.

Attempted preparation of veratraldehyde. To a dry, three-necked flask, equipped with a gas inlet tube, a mechanical stirrer, and a condenser guarded with a drying tube containing anhydrous calcium chloride, were added 3.8 gm of anhydrous stannous chloride and 50 ml of anhydrous ethyl ether. While stirring, dry hydrogen chloride was passed in at room temperature until all the solid stannous chloride had disappeared with the formation of a second liquid layer. This took about thirty minutes.

The addition of hydrogen chloride and the stirring were then interrupted, and a solution of 1.63 gm (0.01 mole) of veratronitrile in 50 ml of dry ethyl ether was added. The additional ether caused some solid stannous chloride to reappear. The stirring was resumed and hydrogen chloride was again passed into the solution. The solution, which still contained two layers, became clear in about ten minutes. After about twenty minutes, a yellow solid began to appear in the lower liquid layer. Finally the whole lower liquid layer disappeared and the resulting yellow solid was suspended in the ether. The stirring and addition of hydrogen chloride were again interrupted and 100 ml of toluene, dried

by distillation from sodium, was added. The ether was distilled from the solution and after saturating again with hydrogen chloride, the resulting toluene solution was refluxed for two hours. During this time, the color of the suspended solid changed from yellow to white. After cooling, the flask was stoppered and placed in the ice box overnight.

The resulting white solid was filtered and washed with dry ethyl ether. This solid was finally decomposed by adding 80 ml of water and heating the resulting solution to 50°C for thirty minutes. An oil appeared, which solidified on cooling. This material was extracted with benzene. After evaporation of the benzene, 1.1 gm of solid was obtained. This solid was purified chromatographically by passing it, in an ether solution, through a magnesol column. After purification, the solid melted 67°C and showed no depression when a mixed melting point with authentic veratronitrile was taken. This was, therefore, a recovery of the starting material, veratronitrile.

This reaction was also attempted with only ethyl ether, with only toluene, and with sym. tetrachloroethane as the solvent. The temperatures were varied between 0°C and the boiling point of the solvent. The reaction was also tried using anhydrous aluminum chloride in addition to the stannous chloride. All the methods tried resulted in good recovery of the starting veratronitrile, rather than the desired

product, veratraldehyde.

Since these experiments with the Stephen's reaction were not successful, attempts were made to convert the nitrile, or one of its derivatives, into veratraldehyde by another method. It was found that veratronitrile, after hydrolysis to veratric acid, could be converted into veratroyl chloride, and this product could be reduced to veratraldehyde by the Rosenmund reaction. This series of reactions is later described in detail in the experiments using labeled material.

Once the aldehyde was prepared, it was to be condensed with dimethylphloroacetophenone and, therefore, this intermediate was prepared.

Preparation of 2,4-dimethylphloroacetophenone

This synthesis necessitated the preparation of phloroacetophenone from the more readily available phloroglucinol.

Preparation of phloroacetophenone. A dry flask was charged with 20 gm (0.16 mole) of phloroglucinol, dried at 120°C, 13 gm (0.32 mole) of anhydrous acetonitrile, 80 ml of anhydrous ethyl ether, and 4 gm of powdered, anhydrous zinc chloride. This flask was fitted with a large-bore gas inlet tube, reaching to the bottom of the flask and a drying tube through which the exhaust gases could leave the vessel. After cooling the mixture to 0°C, dry hydrogen

chloride was passed in at a rapid rate for two hours. A yellow solid appeared during this time. The flask was then stoppered and placed overnight in the ice box.

Dry hydrogen chloride was again passed into the reaction vessel for two hours. The flask was allowed to stand for three days in the ice box.

The resulting solid ketimine hydrochloride was filtered and washed with dry ether. It was then refluxed with one liter of water for two hours. On cooling, the phloroacetophenone appeared as yellow needles. Recrystallization from hot water, using activated charcoal, produced a pale yellow product which weighed 17 gm and had a melting point of 218°C. The partial methylation of this product is described below.

Preparation of 2,4-dimethylphloroacetophenone. In a dry flask, 12.6 gm of phloroacetophenone, dried at 120° C, was dissolved in 45 ml of anhydrous acetone. In the hood, 225 ml of anhydrous benzene, 45 gm of anhydrous potassium carbonate, and 14.5 ml of dimethyl sulfate were added to this solution. This mixture was refluxed on a water bath for twelve hours. Occasional bumping did occur, but this bumping did not get out of control.

The resulting mixture was filtered and the residue washed with hot benzene. The filtrate and benzene washings were then washed with water and extracted with 5% aqueous sodium hydroxide. This basic solution was poured into cold,

25% hydrochloric acid which caused the dimethylphloroacetophenone to separate as white crystals. These crystals were filtered and washed with 5% aqueous sodium carbonate and then with water. After drying in a vacuum desiccator, the product weighed 12 gm and had a melting point of 81°C.

This product was purified further by passing it through a magnsol column. The column was made up with anhydrous ethyl ether. An etheral solution of the dimethylphloroacetophenone was then run through the column. The compound passed through the column with the solvent front. After removing the ether under reduced pressure, the product was dried in a desiccator. The 2,4-dimethylphloroacetophenone melted at 83°C.

Studies on the preparation of the chalcone of completely methylated eriodictyol by the condensation of veratraldehyde with this product, 2,4-dimethylphloroacetophenone, were then undertaken.

Preparation of the chalcone of 5,7,3',4'-tetramethyleriodictoyl

These studies were undertaken to determine the optimum conditions for the condensation of veratraldehyde with dimethylphloroacetophenone. The method used with the labeled material will be described, in detail, in a later section. This method, in general, consisted in reacting equivalent quantities of the aldehyde and ketone in a basic ethanol solution.

A solution containing 2 gm (0.012 mole) of veratraldehyde and 2.4 gm (0.012 mole) of dimethylphloroacetophenone in 75 ml of 95% ethanol was treated with 4.5 ml of 50% aqueous potassium hydroxide. This solution was refluxed for three hours. During this time, the color changed to a very dark red. On dilution with 200 ml of distilled water and acidifying with concentrated hydrochloric acid, a deep orange precipitate was obtained. After filtering, this crude chalcone was washed with dilute ethanol and dried in a desiccator. The product weighed 1.8 gm which is a 43% yield.

In another reaction, the same amounts of reactants were dissolved in 75 ml of 95% ethanol and 20 ml of 50% aqueous potassium hydroxide were added. This solution was allowed to stand at room temperature for two days, during which time the solution turned to a cherry red. Upon diluting the solution with 150 ml of distilled water and acidifying with concentrated hydrochloric acid, the crude chalcone, as a yellow precipitate, was obtained. This product after washing and drying, weighed 2.1 gm and a 51% yield.

Since these methods did not produce yields as high as the method to be described with the labeled material, they were not used.

Preparation of 5,7,3',4'-tetramethyleriodictyol

The preparation of the methylated eriodictyol consisted in closing the heterocyclic ring of the corresponding chalcone. The details used in the labeled syntheses will

be described later. However, two sets of conditions which were not as successful are described.

A 5 gm (0.014 mole) quantity of purified chalcone, dissolved in 500 ml of absolute alcohol, was treated with 25 ml of concentrated hydrochloric acid. This solution was refluxed for ten hours. After cooling, the solution was diluted with 200 ml of distilled water and extracted with three 50 ml portions of benzene. The benzene solution, after drying by azeotropic distillation, was passed through a column of magnesol, which had been prepared with dry benzene. The flavanone, tetramethylated eriodictyol, adhered tightly to the top portion of the column, while the unreacted chalcone developed below this flavanone. By extruding the wet magnesol from the column with forced air, it was then possible to cut out the flavanone and chalcone bands. These bands were then eluted with acetone. After removal of the acetone, the flavanone weighed 1.2 gm (24% yield) and the unreacted chalcone weighed 3.1 gm (62% recovery).

This cyclization procedure was also carried out with 3 gm of chalcone dissolved in 450 ml of 95% ethanol, and treated with 80 ml of a 50% aqueous hydrochloric acid solution. The reflux time was sixteen hours. The flavanone was obtained in a manner similar to that described in the previous reaction. A 38% yield of the eriodictyol was obtained.

Preparation of 5,7,3',4'-tetramethylquercetin

The method described in the labeled synthesis consisted in preparing the oxime of the corresponding eriodictyol, in the 3 position, and hydrolyzing the product to methylated quercetin in one step. Attempts were made to isolate the intermediate oxime and thus obtain a higher conversion.

A solution of 0.25 gm (0.725 millimole) of tetramethyleriodictyol and 1 ml of freshly prepared butyl nitrite in 25 ml of 1,4-dioxane was prepared. A second solution was prepared which contained 0.65 ml (1.5 millimole) of trimethylbenzylammonium hydroxide in 40 ml of dioxane. The resulting cloudy solution was clarified by the addition of 10 ml of absolute ethanol.

The flavanone solution was then added dropwise, with stirring, to the basic solution. The addition took about fifteen minutes, after which an additional 0.5 ml of butyl nitrite was added. The solution remained a pale yellow. The stirring was continued for one hour.

Attempts were made to isolate the oxime from this solution by adding 200 ml of ice water and acidifying with dilute hydrochloric acid. In this manner, 0.1 gm of unchanged eriodictyol was recovered. Extraction of the aqueous solution, before and after acidification, with benzene, or ethyl ether, or with chloroform did not produce any of the oxime. Attempts to precipitate an oxime from the dioxane solution by the addition of sulfuric acid or with ethyl

ether were also unsuccessful.

Preparation of Quercetin-2-C^{14}

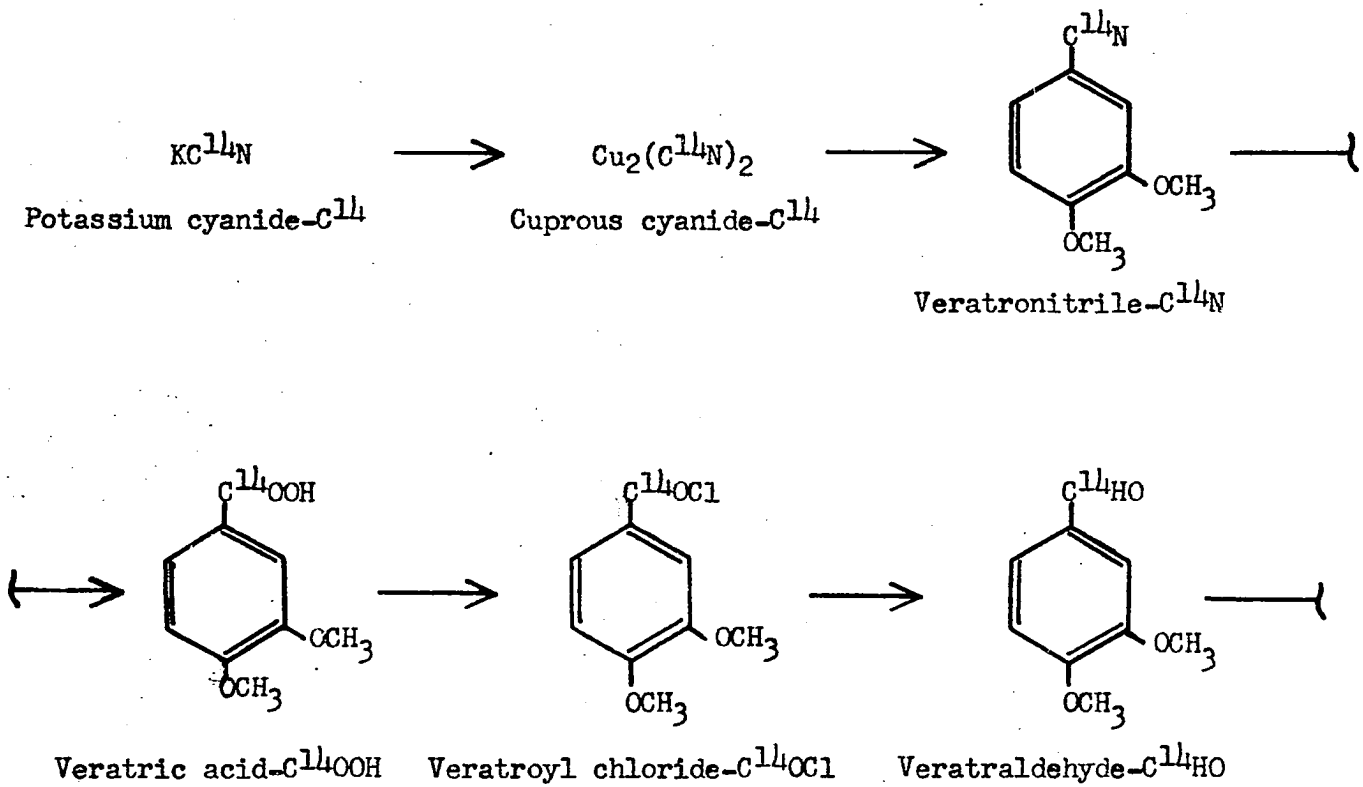
The total synthesis of quercetin-2-C^{14}, using potassium cyanide-C^{14} as the labeled starting material, is described in the following paragraphs and is illustrated in Figures 3 and 4. All the labeled syntheses were first carried out in trial runs using non-labeled material. The assay procedures, for the specific activities given with these syntheses, will be described in later sections.

Dilution of potassium cyanide-C^{14}

The starting potassium cyanide-C^{14} was obtained from Nuclear Instrument and Chemical Corporation (Chicago) in a sealed ampule and had a specific activity of 10.2 millicuries per millimole. One millicurie is equal to 3.7×10^7 disintergrations per second. Since the sealed ampule contained a total of one millicurie, this means that there was 1/10.2 or 0.098 millimoles in the ampule. This is 6.38 mgm of potassium cyanide-C^{14}.

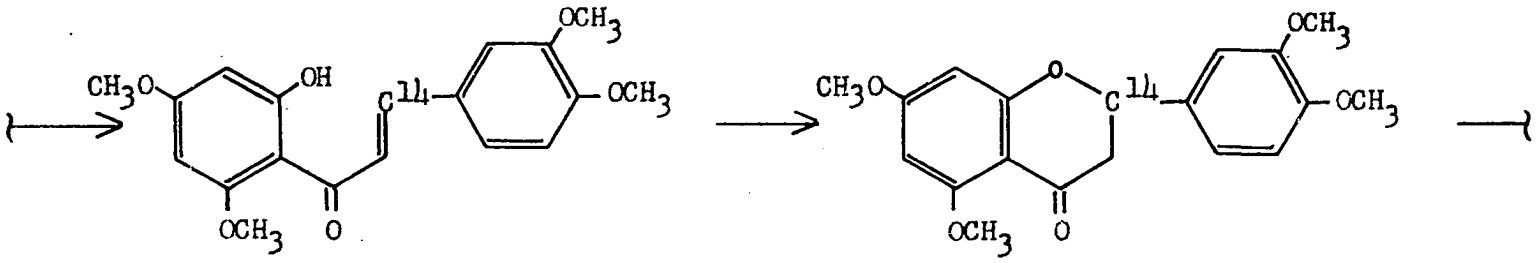
The contents of the ampule appeared as a fine white powder in which the crystals tended to repel each other and adhere to the glass. The ampule was opened by first scoring the glass along the tip with a file and then repeatedly touching the scored area with a hot glass rod. This finally caused the glass to crack, and with a little pressure, the ampule could be opened. This procedure was carried out over

Figure 3.



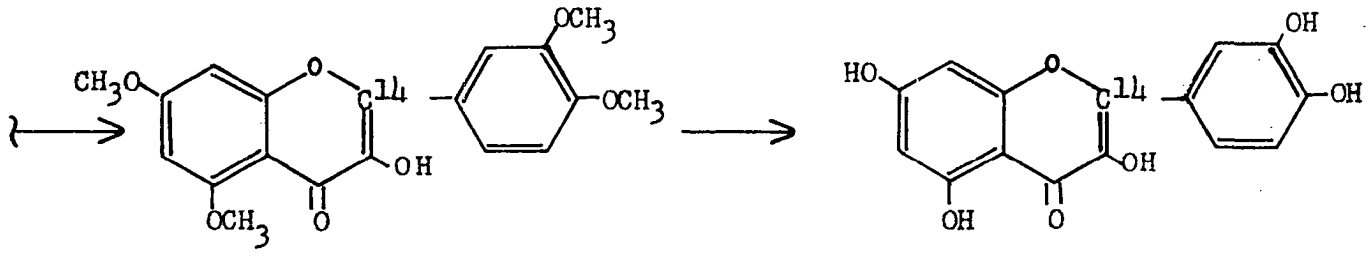
The synthesis of quercetin-2-C^{14}, continuing on Figure 4.

Figure 4.



Chalcone of 5,7,3',4'-tetramethyleriodictyol-2-C^{14}

5,7,3',4'-Tetramethyleriodictyol-2-C^{14}



5,7,3',4'-Tetramethylquercetin-2-C^{14}

Quercetin-2-C^{14}

The synthesis of quercetin-2-C^{14}, continuing from Figure 3.

glassine paper so that in the event of spillage, the active material could be recovered.

The contents of the ampule were then washed into a four inch test tube containing 1.97 gm (0.03 mole) of c.p. potassium cyanide (96.9% pure). The ampule was washed ten times with a total of 5 ml of distilled water. The resulting potassium cyanide contained one millicurie and had, therefore, a specific activity of 0.033 millicuries per millimole. Since further dilutions, with corresponding non-labeled materials, were not made in the following reactions, all intermediates and the final product should have this same specific activity.

Preparation of cuprous cyanide-C^{14}

The test tube containing the 1.97 gm (0.03 mole) of potassium cyanide in 5 ml of water and a test tube containing 2 gm of sodium meta-bisulfite in 5.5 ml of distilled water, were heated in a water bath to 60°C. A solution of 7.14 gm (0.03 mole) of cupric sulfate pentahydrate in 20 ml of distilled water was acidified to congo red paper with dilute sulfuric acid (one drop) and then heated on a water bath to 50°C. The sodium bisulfite was then added slowly to the copper sulfate solution with stirring. The solution changed to a yellowish green color. The potassium cyanide solution was then added, all at once, and the test tube rinsed with two 0.5 ml portions of hot distilled water. The

white cuprous cyanide formed immediately, and, after standing for ten minutes, the warm solution was filtered with suction through a small Buchner funnel. The product was washed with 15 ml of boiling water and then with 20 ml of 95% ethanol.

This product was then transferred into a pre-weighed, standard tapered, ten inch test tube. The product weighed 2.4 gm and represented an 89% yield. On assay it was found to have a specific activity of 0.032 millicuries per millimole.

Preparation of veratronitrile-$C^{14}N$

To the test tube containing the 2.4 gm (0.027 mole) of cuprous cyanide, was added 7.5 gm (0.028 mole) of 4-iodoveratrole. The test tube was then fitted with an air condenser and a stirring rod was inserted through the condenser to the bottom of the tube. The test tube was immersed in an oil bath, which was then heated to a bath temperature of 250°C. This temperature was maintained to ± 10 degrees over a period of two hours and was stirred at frequent intervals by hand. The reaction here seems to be autocatalytic, since after about one hour of heating the solid started to turn gray and the solution began to darken; ten minutes later the solution and solid were very dark. It had been previously noted that with highly purified iodoveratrole, a much longer time was needed for the re-

action to start, if it started at all, than with the more impure material.

During the entire reaction, the solution refluxed on the sides of the test tube. On cooling, the reaction mixture turned to a very hard solid mass. This solid material was then broken up under 15 ml portions of anhydrous ethyl ether with the stirring rod. This took a considerable amount of time, and a total of 60 ml of ether. A dark, fine powder remained in suspension.

A magnesol column was prepared with anhydrous ethyl ether, and after washing with dry ether, the ethereal solution containing the nitrile was passed through the column. The column was finally washed with four columns of dry ether. The eluate, which was a very light tan in color, was then transferred to a pre-weighed, alkali resistant, round bottom flask. After removing the ether under reduced pressure, the residue weighed 4.5 gm. Since this is a 102% yield, the product probably contained some unreacted 4-iodoveratrole. Previous runs with non-labeled material had indicated that this impurity would not hinder the hydrolysis of the nitrile, which followed, and this impurity could then be removed at the later step.

Preparation of veratric acid-C^{14} OOH

To the product from the previous reaction was added 120 ml of a 15% potassium hydroxide solution and 40 ml of

methanol. The flask was fitted with a reflux condenser and the solution was refluxed with a heating mantle for a total of 30 hours. At the end of this period, no more ammonia could be detected with moist litmus paper, at the top of the condenser.

The condenser was then turned down, and the methanol was distilled from the solution. The distillate was basic to litmus. The resulting aqueous solution was then extracted twice with 15 ml portions of ethyl ether to remove any unreacted nitrile. This also removed the iodoveratrole which had been carried over from the preceding reaction. About 0.3 gm of still fairly radioactive impurities were extracted, and were set aside in a glass vial for later reworking.

The acid was finally precipitated by adding concentrated hydrochloric acid to the aqueous solution at 75°C. If the precipitation was made below 50°C, the monohydrate formed, and when dried in an oven, some was lost by sublimation. The light tan crystals of the acid were filtered and washed with distilled water containing a small amount of hydrochloric acid.

Purification was accomplished by dissolving the acid in 25 ml of dilute sodium hydroxide solution and heating this solution with activated charcoal at 100°C. After filtering off the charcoal, the solution was acidified with concentrated hydrochloric acid at 75°C, and the resulting

white crystals were collected by filtration with suction. The product was washed on the funnel with a small amount of dilute acid, and dried in a desiccator.

The veratric acid was then transferred into a pre-weighed, 65 ml, three-necked flask and after drying for thirty minutes at 100°C, weighed 4.0 gm. This is an 80% yield based on the crude nitrile.

After working up the mother liquors and washings, an additional 0.3 gm of veratric acid was obtained. This was set aside in glass vial. The veratric acid melted at 181°C and was assayed to have a specific activity of 0.034 millicuries per millimole.

In order to prove the position of the labeled carbon atom, a 2.4 mgm sample of this acid was taken for decarboxylation. The sample was diluted up to 109 mgm with non-radioactive veratric acid and placed in a glass test tube having a ground glass joint at the top. This test tube was equipped with a gas inlet tube for nitrogen and an exhaust tube which carried the exhaust gases through a U-tube containing 10 ml of 0.1N sodium hydroxide. The sample was reacted with 0.1 gm of cupric chromite in 2 ml of quinoline at an oil bath temperature of 250 ± 10°C. Nitrogen was passed through the system at a slow rate. After thirty minutes of heating, the reaction chamber was cooled and a one molar solution of barium chloride was added to the sodium hydroxide. A white precipitate of barium carbonate formed

immediately. This was filtered, and after washing with water and alcohol, the solid was placed overnight in a dessicator. The barium carbonate was then assayed and a specific activity of 0.0006 millicuries per millimole was observed. Since the dilution factor was 1 to 45, the calculated activity of the barium carbonate was 0.0007 millicuries per millimole.

The residue from the decarboxylation, after drying, had a small residual activity. By heating the residue at 250°C for another two hours, a residue with no detectable activity was obtained.

Preparation of veratroyl chloride-C^{14} OCl

The three-necked flask, containing 4.0 gm (0.022 mole) of veratric acid, was fitted with a condenser, guarded with a calcium chloride tube, and two ground glass stoppers. To this flask was added 25 ml of thionyl chloride. This reagent had been purified by first distilling it from pure quinoline, and then by a second distillation from boiled linseed oil.

A reaction began immediately in the reaction flask. Hydrogen chloride was evolved and after about 10 minutes all the acid had dissolved in the thionyl chloride. The resulting solution had a pale amber color. The flask was then heated on a water bath and the thionyl chloride was refluxed for a period of two hours.

At the end of this time, the water was drained from

the condenser, and a strong water-aspirator was attached to the top of the drying tube with pressure tubing. The excess thionyl chloride was then taken off under suction at a final bath temperature of 70°C. After one hour, the water-aspirator was replaced with a mechanical oil pump, and the last trace of thionyl chloride were removed at about 1 mm of mercury pressure.

The pale amber liquid solidified on cooling. No attempt was made to weigh this product.

Preparation of veratraldehyde-$C^{14}HO$

The veratroyl chloride obtained above was reduced to the corresponding aldehyde by the Rosenmund reaction.

Purification of hydrogen. Previous runs with unlabeled material had shown that the commercial hydrogen must be freed of small traces of oxygen, in order to obtain optimum yields. This was accomplished by bubbling the hydrogen through Fieser's solution.[48] This solution was prepared by dissolving 20 gm of potassium hydroxide in 100 ml of water, and adding 2 gm of sodium anthraquinone-β-sulfonate and 15 gm of sodium hyposulfite to the warm solution. The deep red solution was stirred until all the salts had dissolved. A change in color to brown or the formation of a precipitate indicates that the solution had become inactive.

The hydrogen, after passing through the Fieser's

solution, was dried by bubbling it through concentrated sulfuric acid. Some silver sulfate was added to the sulfuric acid to detect any hydrogen sulfide that might be formed by the Fieser's solution.

Purification of the solvent. Xylene was chosen as the solvent, since at its boiling point, the reaction proceeds smoothly at a slow rate. At higher temperatures the reaction was too vigorous, and the resulting aldehyde was reduced further to the corresponding alcohol. At lower temperatures the reaction did not take place.

The xylene used was purified by distillation from sodium.

Preparation of the catalyst. The catalyst used in the Rosenmund reaction was palladium on barium sulfate.

A solution of 4.4 gm of anhydrous sodium sulfate in 60 ml of water was added dropwise, with stirring, to a solution of 6.2 gm of barium chloride dihydrate in 60 ml of water at 70°C. The precipitate of barium sulfate which formed was then washed by decantation with hot water until the washings gave no precipitate with aqueous silver nitrate. The barium sulfate was then suspended in 90 ml of water containing 5 drops of aqueous formaldehyde and heated to 80° C.

A solution of 0.5 gm of palladium chloride in 30 ml of water, which had stood at room temperature for three days, was then added. The resulting solution was rendered neutral

to litmus, slowly, with 1 N sodium hydroxide. The heating and stirring were continued for thirty minutes. The gray powder was then allowed to settle and was washed by decantation until no more chloride ion was detected with aqueous silver nitrate.

The precipitate was then filtered with suction and dried in a desiccator over calcium chloride. The resulting catalyst weighed 6.5 gm and should contain about 5% palladium.

Preparation of the regulator. In order to keep the aldehyde from being reduced to the alcohol after it was formed, a small amount of a "poison" was added to the reaction mixture. The regulator was quinoline-sulfur and was prepared by refluxing 1 gm of sulfur in 6 ml of freshly distilled quinoline. After cooling, the deep brown-red solution was diluted with 70 ml of purified xylene and stored in a glass stoppered bottle.

Preparation of the aldehyde. The three-necked reaction flask containing the veratroyl chloride was now equipped with a gas inlet tube, which was connected to the hydrogen source (sulfuric acid wash bottle) with tygon tubing. From the water condenser, provisions were made to lead the exhaust gases through a drying tube into a graduated cylinder containing distilled water and phenophthalein as an indicator. By adding 0.1N sodium hydroxide to the distilled water, it was possible to tell the relative speed of the reaction by the rate of evolution of hydrogen chloride.

The reaction was terminated when the phenophthalein was no longer decolorized.

The reaction flask was immersed in an oil bath which was heated by a hot plate containing a magnetic stirrer. To this flask, containing 0.022 mole of veratroyl chloride, based on the veratric acid, was added 30 ml of purified xylene, 5 microliters of the quinoline-sulfur regulator and a quarter inch magnetic stirring bar. The system was then heated to a bath temperature of 60°C and flushed thoroughly with hydrogen.

After stopping the flow of hydrogen, the gas inlet tube was removed and 0.5 gm of the palladium-barium sulfate catalyst was added. Care was taken that the hydrogen flow had ceased, since the catalyst would spontaneously ignite in the presence of the hydrogen.

The gas inlet tube was re-inserted and the hydrogen was again started at the rate of about one bubble per second. The oil bath temperature was raised to 160°C and the solution was stirred vigorously.

At the end of sixteen hours the exhaust gases would no longer decolorize the phenophthalein and the reaction was terminated. The resulting solution was filtered into a 250 ml flask. All the equipment was rinsed with absolute ethanol and with anhydrous ethyl ether. These washings were added to the aldehyde solution. The solvents from this flask were then removed under reduced pressure. The resulting

residue, which was not purified, was light yellow in color and was carried on to the next reaction. It weighed about 3.6 gm.

At the end of the next reaction, 0.68 gm of unreacted veratraldehyde was recovered. This gave a specific activity of 0.033 millicuries per millimole.

Preparation of the chalcone of 5,7,3',4'-tetramethyleriodictyol-2-C^{14}

The residue from the preceding aldehyde preparation was assumed to contain about 2.2 gm (0.012 mole) of veratraldehyde. This assumption was based on previous runs with unlabeled material which had shown that a maximum yield of 60% of veratraldehyde could be expected from the veratroyl chloride.

This residue and 3.0 gm (0.015 mole) of 2,4-dimethylphloroacetophenone were dissolved in a minimal amount of 95% ethanol. This took about 200 ml. To this solution, 6 ml of 50% aqueous potassium hydroxide was added. The reaction vessel was then shaken at frequent intervals over a thirty minute period, and the solution changed in color from amber to orange. The flask was then stoppered and placed on top of a hot air oven at about 40°C for forty-eight hours.

At the end of this time, the solution, which had become a very deep red, was diluted with 400 ml of distilled water. Concentrated hydrochloric acid was added dropwise

to the solution until it showed an acid reaction to congo red paper. A flocculent yellow precipitate appeared. This was placed in the ice box overnight.

The precipitate was filtered without suction and the filtrate was extracted with benzene until no more color was removed. This took four 50 ml portions of benzene. This 200 ml of benzene was then used to dissolve the precipitate and the resulting solution was distilled for fifteen minutes. This action dried the benzene by azeotropy and was necessary, since, from wet benzene, the solutes would not be readily adsorbed on a magnesol column.

A column of magnesol was prepared and washed with anhydrous benzene. The crude chalcone solution was then run through this column. The chalcone developed as a bright yellow band near the top of the column. Under ultraviolet light this band appeared as a dark brown florescence. The first portions of eluant from this column were colorless and were collected in 25 ml quantities.

The first 125 ml of eluant yielded, after evaporation of the benzene, 1.6 gm of unreacted tetramethylphloroacetophenone, which had a sharp melting point at 83°C. A clear separation between this ketone and the aldehyde which followed it on elution was not accomplished, and the last portion of the ketone possessed some activity.

The unreacted veratraldehyde, which followed the phloroacetophenone from the column, was collected in the

next 75 ml of eluant. On evaporation of the benzene, a yellow oil was obtained. This oil was dissolved in ethyl ether and passed through a magnesol column, which had been prepared with anhydrous ether. The ether was allowed to evaporate from the eluant and 0.68 gm of crystalline veratraldehyde was obtained. This was set aside in a glass vial.

The chalcone, by this time, had developed as a band below the dark impurities at the top of the column and below a narrow pale yellow band just under the impurities. After washing with a total of two columns of anhydrous benzene, the wet magnesol was extruded from the top of the column. This was accomplished by applying air pressure to the constricted lower end of the column. By holding the column horizontally, one inch above a stainless steel tray, and applying the pressure very slowly, it was possible to ease the magnesol from the top of the column. In this manner, the magnesol retained its cylindrical shape. The magnesol was then cut with a stainless steel spatula into the three bands. The top band contained the impurities and was set aside. The second band, which had been shown in previous runs to contain some tetremethyleriodictyol, was eluted with anhydrous acetone. This was combined with the crude eriodictyol obtained in a later reaction.

The third band contained the purified chalcone and was eluted with anhydrous acetone. The eluant was filtered into a 500 ml round bottom flask and the solvent was removed

under reduced pressure. The chalcone weighed about 2.3 gm. This is a 30% yield based on the veratric acid.

At the end of the next reaction 0.44 gm of chalcone was recovered. It was assayed to have a specific activity of 0.034 millicuries per millimole.

Preparation of 5,7,3',4'-tetramethyleriodictyol-2-C^{14}

The 2.3 gm (0.0067 mole) of chalcone from the preceding reaction was dissolved in 300 ml of 95% ethanol. After adding 11 ml of concentrated hydrochloric acid in 30 ml of distilled water, the resulting solution was refluxed for twenty hours.

At the end of this time, 500 ml of distilled water was added and a bright yellow precipitate formed. The precipitate was filtered without suction and the filtrate was extracted three times with a total of 150 ml of benzene. This benzene was then used to dissolve the precipitate. The resulting solution was filtered to remove the drops of water present and then dried carefully by azeotropic distillation.

A column of magnesol was prepared and washed with anhydrous benzene. The flavanone solution was then passed onto the column. The flavanone was adsorbed tightly onto the adsorbent and appeared an ivory color in visible light and a dull gray in ultraviolet light. The chalcone normally developed just below the flavanone. In this case the column

was not quite long enough to hold all the flavanone and, as a result, all of the chalcone and a small amount of the flavanone passed off the column. It took 250 ml of anhydrous benzene to wash the chalcone from the column. The column which now contained only the flavanone and a small amount of impurities on the top surface, was washed with 300 ml of anhydrous acetone. This removed the flavanone very readily. The first portion of acetone through the column carried some of the flavanone and made a cloudy solution with the benzene. The flavanone solution was set aside and combined later with some more of the flavanone.

The chalcone eluate that was obtained above, was evaporated to dryness under reduced pressure. The resulting orange crystals weighed about 1.5 gm.

This recovered chalcone was recycled through the same reaction. The 1.5 gm of chalcone was dissolved in 200 ml of 95% ethanol containing 7 ml of concentrated hydrochloric acid and 20 ml of water. After refluxing for twenty hours, the solution was diluted with water and taken up with benzene as described previously. The benzene solution, after drying, was passed onto a fresh magnesol column. This time the flavanone did not saturate the column and some of the bright yellow chalcone remained adsorbed below the ivory colored flavanone. This chalcone could not be washed off the column with dry benzene. Therefore, the column was extruded as described in the chalcone preparation. The flavanone

band was removed and after elution with acetone, was combined with the flavanone obtained earlier.

The chalcone band was also removed and eluted with acetone. This was combined with the eluate that had come through the column. After removal of the solvent it weighed about 0.8 gm.

This chalcone was recycled for a second time through the same procedure. After separating the products on a magnesol column, the recovered chalcone weighed 0.44 gm and was set aside in a glass vial. The flavanone obtained was combined with that already on hand. The solvent was removed from the combined solutions and the flavanone was obtained as a pale yellow solid which weighed 1.5 gm. This is a 66% conversion of the chalcone.

Preparation of 5,7,3',4'-tetramethylquercetin-2-C^{14}

The methylated eriodictyol from the preceding reaction was then converted by means of n-butyl nitrite into the oxime, which was hydrolysed to the corresponding flavonol, quercetin. Therefore, the preparation of butyl nitrite was necessary and was freshly prepared before each reaction.

Preparation of n-butyl nitrite. In the hood, a round bottom flask was equipped with a stirrer. A dropping funnel extending to the bottom of the flask was also used. After placing this flask in an ice-salt bath, 47 gm of sodium nitrite and 160 ml of distilled water were added. The

stirring was started, and when the temperature reached 0°C, a solution containing 12 ml of water, 17 ml of concentrated sulfuric acid and 70 ml of n-butyl alcohol, was added slowly from the dropping funnel. The temperature was maintained below 3°C and the addition took about forty-five minutes. The mixture, containing a solid and two liquid layers, was left standing for two hours, and then filtered. The upper layer of the filtrate, containing the butyl nitrite, was separated and washed with a solution of 0.6 gm of sodium bicarbonate and 6 gm of sodium chloride in 25 ml of water. The butyl nitrite, after drying over magnesium sulfate, was distilled at 30°C under the reduced pressure of a water aspirator and yielded 40 ml.

Conversion of the flavanone to the flavonol. The 1.5 gm of eriodictyol was dissolved in 100 ml of boiling 95% ethanol. To this boiling solution was added, alternately and in small portions, 10 ml of n-butyl nitrite and 15 ml of concentrated hydrochloric acid. The addition required about ten minutes and the heating was continued for another ten minutes. The solution was allowed to cool and stand at room temperature overnight.

This mixture was then diluted with 400 ml of water and caused the tetramethyl quercetin to precipitate. After twelve hours, the precipitate was filtered and washed with 10% ethanol. On drying, 0.15 gm of the flavonol was obtained.

The filtrate and washings were extracted with benzene.

After drying this solution, the benzene was removed under reduced pressure, and a semi-solid residue was obtained. This weighed 0.7 gm and was actually the solid flavonol and an oil. The solid was recovered by dissolving the oil in 50% ethanol and filtering the flavonol. The methylated flavonol obtained weighed 0.3 gm.

Preparation of quercetin-2-C^{14}

The two samples of methylated quercetin obtained in the previous reaction, had been collected and dried in 50 ml centrifuge tubes and were demethylated separately.

The tube containing the 0.15 gm of methylated flavonol was carefully made into a paste with 5 drops of acetic anhydride. Then 10 ml of hydriodic acid and two boiling chips were carefully added. This caused a little spattering. This acid was a Merck product and contained hypophosphorus acid as a preservative. The test tube, fitted with a condenser, was then immersed in an oil bath and refluxed. A precipitate appeared in about thirty minutes. At the end of two and a half hours, the heating was stopped and the solution was diluted with 30 ml of distilled water. After thorough mixing, the tube was jacketed with a stainless steel tube and centrifuged. In this case the glass centrifuge tube broke. However, the contents were saved by the metal jacket and were recovered by rinsing with ethyl acetate. The resulting ethyl acetate solution after washing with water, was

passed through a magnesol column. The quercetin passed through quite rapidly. By evaporating the ethyl acetate from the eluate, 0.1 gm of quercetin was obtained.

The second tube of methylated quercetin (0.3 gm) was demethylated in a similar manner. Ten drops of acetic anhydride and 15 ml of hydriodic acid were used. The precipitate which formed was centrifuged and then washed three times with distilled water. After each washing, the quercetin was re-centrifuged. The product after drying weighed 0.2 gm. The overall conversion of labeled potassium cyanide into quercetin was 3.5%. Previous runs with unlabeled material had given an 8% yield. During the synthesis with unlabeled material, intermediates were recovered to the extent of 11% of the starting potassium cyanide. In the labeled syntheses, a 29% recovery of intermediates, based on the labeled potassium cyanide, were obtained.

The labeled quercetin was run on paper chromatograms in comparison with unlabeled synthetic quercetin, and with unlabeled quercetin isolated in pure form from buckwheat. 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-butanol-acetic acid-water, 6:1:2, and 60% aqueous acetic acid.

In order to show the position of the labeled carbon atom, 1.3 mgm of this quercetin was taken for degradative analysis. This was diluted with 68.5 mgm of unlabeled

quercetin, which made a total of 69.8 mgm. The diluted quercetin had a calculated specific activity of 0.00055 millicuries per millimole.

This quercetin was first methylated. The 69.8 mgm of quercetin was dissolved in 35 ml of anhydrous acetone, a 5 gm of anhydrous potassium carbonate was then added to the yellow solution. The flask was then fitted with a condenser, through which 1 ml of dimethyl sulfate was added. The reaction mixture was refluxed. Some bumping did occur but this did not get too serious. The solution lost all its color in about three hours. An additional 1 ml of dimethyl sulfate was added, and the refluxing was continued for another three hours to insure complete methylation.

The solution was filtered and the filtrate was taken to dryness on a steam bath. The oil which resulted was dissolved in ethanol. The addition of water caused the separation of some white needles. On drying, the crystals of pentamethyl quercetin melted at 147°C. This melting point was not depressed when a mixed melting point was taken with an authentic sample of unlabeled pentamethyl quercetin. On assay, the labeled pentamethyl quercetin was found to have a specific activity of 0.0005 millicuries per millimole.

The pentamethyl quercetin, which weighed about 70 mgm, was then treated with 15 ml of a solution containing 4 gm of potassium hydroxide in 45 ml of absolute ethanol. This solution, on refluxing, turned yellow and finally brown.

After eight hours, the condenser was removed and the ethanol distilled off. The resulting solid was dissolved in 10 ml of water. Concentrated hydrochloric acid was added to render the solution acidic.

This acidic solution was extracted with three 10 ml portions of ethyl ether. Previous trial runs with unlabeled material had shown that this ether solution contained an acid fragment, veratric acid, and a ketone fragment. It had also been indicated, by use of paper chromatography, that these two fragments could be separated rather cleanly by extracting the ether solution with a 5% sodium carbonate solution.

Therefore, the ether solution, containing the labeled material, was extracted with two 10 ml portions of a 5% sodium bicarbonate solution. The acid portion was extracted by this bicarbonate solution. The ketone fragment, which remained in the ether, was obtained as an oil which did not contain any detectable radioactivity.

The bicarbonate solution, containing the acid, was then rendered acidic with concentrated hydrochloric acid. After standing over night, about 10 mgm of veratric acid was collected. This had a melting point of 179°C and assayed to have a specific activity of 0.0005 millicuries per millimole.

The veratric acid was then decarboxylated in a manner similar to that previously described. The acid was placed

in a small test tube containing 15 mgm of copper chromite in 0.5 ml of purified quinoline. The exhaust gases were led into a 0.1N sodium hydroxide solution, which collected the liberated carbon dioxide-C^{14}. After four hours of heating at 260°C, the carbonate was precipitated from the basic solution using barium chloride. The quinoline residue did not contain any detectable radioactivity.

The barium carbonate was filtered in a small Buchner funnel and washed with 5 ml of water and then with 10 ml of ethanol. The product after drying in a desiccator, was assayed to have a specific activity of 0.0004 millicuries per millimole.

Radioactive Assays and Procedures

The instrumentation and assay procedures used in this work are described below.

Instrumentation

The instrumentation used in assaying for radioactivity consisted of a mica end-window Geiger-Mueller tube as the counter, mounted in a lead shield over a suitable sample holder, and an automatic scaling unit. This apparatus was set up in a room where only radioactive assays were made.

Efficiency of the system

The efficiency of the system was determined by the following formula using a standard source:

$$\frac{\text{(Recorded counts per minute) - (Background)}}{\text{Disintegrations per minute of the standard source}}$$

This is a ratio of the number of counts the system will detect to the total number of particles emitted from the source.

The standard source was a sample of solid barium carbonate-C^{14} obtained from Nuclear Instrument and Chemical Corporation (Chicago) and had a specific activity of 18.6 0.3 disintegrations per second per mgm. Since all of the samples were run with a thickness of less than 1 mgm/cm^2, the standard barium carbonate was also run in this range. No corrections were made for self-absorption by the samples. All the samples were run in similar plancets, machined stainless steel cups, with a measured surface area of 4.7 cm^2.

The plancet was first weighed and then a suspension of the standard carbonate-C^{14} in absolute ethanol was added and spread as uniformly as possible over the plancet with a pyrex stirring rod. The alcohol was allowed to evaporate and the plancet was reweighed after drying. The increase in weight was 1.04 mgm which is an average thickness of 0.222 mgm/cm^2. The sample was counted over a period of 40 minutes and 1259 counts were obtained. This is 31.47 counts per minute per 1.04 mgm and includes the background count.

The background, or stray radiation, was also taken each time a sample was assayed. An empty plancet was placed in the sample holder and the counts were recorded over the

same period of time as that used for the sample.

At the time the standard was assayed, the background was measured as 429 counts over a 40 minute period or 10.72 counts per minute. By subtracting the background count from the sample count a value of 20.75 counts per minute per 1.04 mgm was obtained for the standard. This is a counting rate of 19.95 counts per minute per mgm.

The efficiency of this system was, therefore, 19.95 divided by (18.6 x 60) or 0.0179 (1.79%).

Statistical accuracy

All samples were counted to give a probable error accuracy of 2%. This is also a reliable error accuracy of 5%. To do this, a total of more than 1100 counts must be taken on each sample.

Coincidence losses

At higher counting rates, statistical errors decrease, but errors due to coincidence losses increase. Since some of the samples to be counted had a high counting rate, the resolution time for the counting system was determined. Two events entering the counter within this time interval produces only one pulse, since the second particle enters the counter before most of the positive ions of the previous discharge are collected.

In this procedure, one sample was counted for 60 minutes and found to have a counting rate of 4140 counts

per hour. A second sample was then placed near the counter, without moving or blocking the position of the first sample. The two samples were counted for 60 minutes and a value of 6773 counts was observed. The first sample was then removed without changing the position of the second sample and this sample was measured as 3317 counts per hour. The background was measured over an equal length of time and a value of 683 counts per hour was recorded.

If one lets A and B be the counting rates, in counts per minute, of the first and second sample, respectively, and C be the counting rate observed for both samples, then the resolution time, T, in seconds, may be approximated by the formula:

$$T = \frac{A + B - C}{2AB} \times 60 = \frac{57.61667 + 43.9 - 101.5}{2(57.61667)(43.9)} \times 60$$

When this equation is solved using the data given above, a resolution time of 198 microseconds was obtained.

The loss of counts due to this phenomena is called the coincidence loss. The counter is insensitive for a time equal to NT/60 minutes during each minute, where N is the recorded counting rate in counts per minute and T is the resolution time in seconds. The percentage of counts lost is equal to the percentage of time the counter is insensitive, or NT/60 x 100%.

The use of these equations may be illustrated by taking the counting rate of veratric acid-$C^{14}OOH$ which was

determined to be 17,592 counts per minute per 2.48 mgm. The percentage of counts lost is therefore, (17,592)(0.000198)/ 60 x 100% or 5.8% of the counts. This amounts to a loss of 1,020 counts so that the corrected count of the veratric acid would be 18,612 counts per minute per 2.48 mgm. If this is divided by the efficiency of the counting system, 1.79%, the true rate of 1.04×10^6 disintegrations per minute per mgm or 6.98×10^3 disintegrations per second per mgm is obtained. Multiplying by 182.2, the number of mgm in a millimole of veratric acid, and dividing by 3.7×10^7, the number of disintegrations per second in a millicurie, one obtains 0.034 millicurie per millimole as the specific activity of the veratric acid.

Laboratory monitor

In addition to the counting system mentioned above, a laboratory monitor with a mica end-window Geiger-Mueller tube as a probe, was set up in the laboratory where the syntheses took place. This was used in following the active materials and to check for contamination.

CHAPTER III

SUMMARY

The use of radioactive isotopes, as a tool to aid in elucidating the fate of flavonoids in biological systems, has been undertaken by Wender and co-workers.

As part of the preliminary radioactive work in these studies, this thesis reports on the first synthesis of a specifically labeled polyhydroxy flavonoid. The flavonoid prepared was quercetin-2-C^{14}.

Preliminary investigations on the total synthesis of quercetin were undertaken and are described. From the reactions studied, those which proved to be most feasible were used in the syntheses involving radioisotopes. The starting material was potassium cyanide-C^{14} and the following reactions were carried out.

1. Potassium cyanide-C^{14} was converted into cuprous cyanide-C^{14}.

2. The cuprous cyanide-C^{14} was reacted with 4-iodoveratrole to produce veratronitrile-$C^{14}N$.

3. Veratronitrile-$C^{14}N$ was hydrolyzed to veratric acid-$C^{14}OOH$.

All the radioactivity was shown to be in the carboxyl group of the veratric acid by decarboxylation of this product with copper chromite. Radioactive assays of the residue and of the liberated carbon dioxide-C^{14} were made.

4. Veratric acid-$C^{14}OOH$ was converted, with thionyl chloride, into veratroyl chloride-$C^{14}OCl$.

5. The veratroyl chloride-$C^{14}OCl$ was reduced, by the Rosenmund reaction, into veratraldehyde-$C^{14}HO$.

6. Veratraldehyde-$C^{14}HO$ was condensed with 2,4-dimethylphloroacetophenone, in a basic solution, to produce the chalcone of 5,7,3',4'-tetramethyleriodictyol-2-C^{14}.

7. A ring closure of the chalcone of 5,7,3',4'-tetramethyleriodictyol-2-C^{14} was accomplished in an acidic solution. The 5,7,3',4'-tetramethyleriodictyol-2-C^{14} was obtained.

8. The conversion of the 5,7,3',4'-tetramethyleriodictyol-2-C^{14} into its 3-isonitroso derivative with n-butyl nitrite and the hydrolysis of this product with hydrochloric acid into 5,7,3',4'-tetramethylquercetin-2-C^{14} was carried out in one step.

9. The demethylation of 5,7,3',4'-tetramethylquercetin-2-C^{14} was accomplished with hydriodic acid. The final product quercetin-2-C^{14} was obtained.

As a proof of its structure, this product was completely methylated and the resulting derivative checked

with an authentic sample of pentamethylquercetin. In addition the pentamethylquercetin-2-C^{14} was degraded by rupture of the heterocyclic ring in an alkaline solution. The product of this degradation, veratric acid-2-C^{14}, was decarboxylated with copper chromite and radioactive analyses showed that all the activity was liberated as carbon dioxide-C^{14}.

The instrumentation used to assay the radioactivity in these syntheses was set up and calibrated. The assay procedures are described.

BIBLIOGRAPHY

1. Hevesy, G., Biochem. J., 17, 439 (1923).

2. Glascock, R., Labelled Atoms, Sigma Books Limited, London, 1951.

3. Hevesy, G., Radioactive Indicators, Interscience Publishers, New York, 1948.

4. Sacks, J., Isotopic Tracers in Biochemistry and Physiology, McGraw-Hill Book Company, Inc., New York, 1953.

5. Siri, W. E., Isotopic Tracers and Nuclear Radiations, McGraw-Hill Book Company, Inc., New York, 1949.

6. Wolstenholme, G. E. W., Editor, Isotopes in Biochemistry, The Blakiston Company, New York, 1950.

7. Kamen, M. D., Radioactive Tracers in Biology, 2nd edition, Academic Press Inc., New York, 1951.

8. Jones, W. M., Phys. Rev., 76, 885 (1949).

9. Ruben, S. and Kamen, M. D. Phys. Rev., 59, 349 (1941).

10. Shemin, D. and Rittenberg, D., J. Biol. Chem., 153, 401 (1944).

11. Rittenberg, D. and Block, K., J. Biol. Chem., 160, 417 (1945).

12. Szent-Gyorgyi, A. and Rusznyák, St., Nature, 138, 27 (1936).

13. Matusis, I. I., Bull. Biol. Med. Exptl. U.R.S.S., 4, 481 (1937); C. A., 34, 5124.

14. Moll, T., Klin. Wochschr., 16, 1653 (1937); C. A., 32, 3461.

15. Zilva, S. S., Biochem. J., 31, 915 (1937).

16. Geissman, T. A. and Hinreiner, E., Botan. Rev., 18, 77 (1952).

17. Griffith, J. Q., Jr., Krewson, C. F. and Naghski, J., Rutin and Related Flavonoids, Mack Publishing Company, Easton, Pa., 1955.

18. Miner, R. W., Editor, Bioflavonoids and the Capillary, Ann. N. Y. Acad. Sci., Vol. 61, Art. 3, July, 1955.

19. Scarborough, H. and Bacharach, A. L., Vitamins and Hormones, Vol. 7, p. 1, Academic Press Inc., New York, 1949.

20. Seshadri, T. R., J. Ind. Chem. Soc., Ind. and News Ed., 11, 35 (1948).

21. Sevin, A., Compt. Rend. Acad. Sci. Paris, 216, 505 (1943).

22. Fuhrman, F. A. and Crismon, J. M., J. Clin. Invest., 27, 364 (1948); C. A., 45, 6751.

23. Rekers, P. E. and Field, J. B., Science, 107, 16 (1948).

24. Eddy, W. H. and Sokoloff, B., Fed. Proc., 11, 441 (March 1952).

25. Schoenkerman, B. B. and Justice, R. S., Ann. Allergy, 10, 138 (1952).

26. Boines, G. J., Ann. N. Y. Acad. Sci., Vol. 61, Art. 3, July 1955.

27. The American Druggist, p. 33, Jan. 2, 1956.

28. Griffith, J. Q., Jr., Krewson, C. F. and Naghski, J., loc. cit., reference 17, pp. 177-8.

29. Thomson, O. L., Biochem. J., 20, 73 (1926); 20, 1026 (1926).

30. Eddy, W. H. and Sokoloff, B., Fed. Proc., 10, 381 (1951).

31. Wilson, C. W., J. Amer. Chem. Soc., 61, 2303 (1939).

32. Bradford, R. H., M. S. Thesis, University of Oklahoma, 1954.

33. Savage, W. L., M. S. Thesis, University of Oklahoma, 1953.

34. Szent-Györgyi, A., Ann. N. Y. Acad. Sci., Vol. 61, Art. 3, July, 1955.

35. Gage, T. B. and Wender, S. H., Fed. Proc., 8, 293 (1949).

36. Gage, T. B., Douglass, C. D. and Wender, S. H., Anal. Chem., 23, 1582 (1951).

37. Wender, S. H. and Gage, T. B., Science, 109, 287 (1949).

38. Griffith, J. Q., Jr., Krewson, C. F. and Naghski, J., loc. cit., reference 17, p. 242.

39. Kostanecki, St., Lampe, V. and Tambor, J., Ber., 37, 1402 (1904).

40. Kostanecki, St. and Tambor, J., Ber., 37, 792 (1904).

41. Fox, D. W., private communication.

42. Barger, G. and Silverschmidt, R., J. Chem. Soc., 131, 2919 (1928).

43. Adams, R. and Levine, I., J. Amer. Chem. Soc., 45, 2373 (1923).

44. Arnold, R. T. and Sprung, J., J. Amer. Chem. Soc., 60, 1699 (1938).

45. Shriner, R. L. and Fuson, R. C., The Systematic Identification of Organic Compounds, 3rd ed., John Wiley & Sons, Inc., New York, 1948.

46. Clark, E. P., J. Amer. Chem. Soc., 53, 3434 (1931).

47. Stephen, H., J. Chem. Soc., 133, 2786 (1930).

48. Vogel, A. I., A Textbook of Practical Organic Chemistry, Longmans, Green and Co., New York, 1951.