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# STUDIES ON THE ANALYSIS FOR AND THE PREPARATION OF FLAVONOID COMPOUNDS

A Doctoral Thesis

Submitted to

THE UNIVERSITY OF OKLAHOMA

Carl D. Douglass

Date Issued

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## PREFACE

This report is reproduced from a thesis submitted to the University of Oklahoma in partial fulfillment of the requirements for the doctoral degree. The report describes research carried out at the Department of Chemistry, University of Oklahoma under the direction of Professor Simon H. Wender and also research performed subsequently in the Organic Group, Chemistry Division of Oak Ridge National Laboratory under the direction of Dr. O. Kenton Neville.

The research performed at the University of Oklahoma was supported in part by the United States Public Health Service and the Office of Naval Research. The research carried out at Oak Ridge National Laboratory was supported by the Graduate Fellowship program sponsored by the Oak Ridge Institute of Nuclear Studies. This latter fellowship program was guided by a committee consisting of Professor Simon H. Wender, Dr. O. Kenton Neville, Dr. G. R. Noggle, Biology Division of Oak Ridge National Laboratory and Dr. Marion T. Clark of the Oak Ridge Institute of Nuclear Studies.

The thesis from which this report was taken was approved for the University of Oklahoma by a committee consisting of Professors Simon H. Wender, Bruce Houston, J. C. Colbert, Lawrence M. Rohrbaugh, and Harriet Harvey.

TABLE OF CONTENTS


| | Page |
|---|---|
| LIST OF ILLUSTRATIONS | v |
| LIST OF TABLES | vi |
| Chapter | |
| I. INTRODUCTION | 1 |
| II. HISTORICAL | 5 |
| III. EXPERIMENTAL | 30 |
| IV. DISCUSSION | 70 |
| BIBLIOGRAPHY | 94 |

## LIST OF ILLUSTRATIONS


Figure Page

1. Structures of Some Flavonoid Compounds. . . . . 3

2. Absorption Spectrum of Substance Isolated from Big Bend Loco Weed. . . . . 37

3. Absorption Spectrum of Liquiritigenin-2,4-dinitrophenylhydrazone. . . . . 48

4. Absorption Spectrum of Hesperitin-2,4-dinitrophenylhydrazone. . . . . 49

5. Absorption Spectrum of 7-Hydroxyflavanone-2,4-dinitrophenylhydrazone. . . . . 50

6. Absorption Spectrum of 2,4-Dihydroxyacetophenone and Substance from Expt. 9. . . . . 59

7. 2,4-Dinitrophenylhydrazones of Naringin and Homoeridictyol. . . . . 81

## LIST OF TABLES


| Table | | Page |
|---|---|---|
| I. | Chemical Names of Flavonoid Compounds | 6,7 |
| II. | $R_f$ Values of Flavonoid Compounds | 32,33 |
| III. | Properties of 2,4-Dinitrophenylhydrazones of Some Flavanones | 51 |
| IV. | Results of Friedel-Crafts Acylation Studies | 55,56 |
| V. | Conditions and Results of Exchange Reactions on $\alpha,\beta$ Unsaturated Compound | 63 |
| VI. | Conditions and Results of Exchange Reaction with 2,4-Dinitrostilbene | 65 |
| VII. | Attempted Exchange Reactions of Cinnamic Acid | 68 |

# STUDIES ON THE ANALYSIS FOR AND PREPARATION OF FLAVONOID COMPOUNDS

## CHAPTER I

## INTRODUCTION

The flavonoid compounds comprise a very important class of plant pigments which are widely distributed in the vegetable kingdom. The results of studies in the qualitative analysis for and the preparation of members of this group are reported in this thesis.

Studies on the origin and function of naturally-occurring flavonoid compounds have been retarded by the lack of good methods of analysis for and separation of the compounds. Since the pigments usually occur in trace quantities as mixtures of compounds, the problem is difficult. If the metabolism of these compounds is to be understood by interested scientists, it is imperative that adequate analytical tools for the identification and analysis of a given constituent and methods for its isolation be available.

Although flavonoids have not yet been proved to be an integral part of animal tissue, considerable interest, nevertheless, has been shown recently in a vitamin-like action of several of the compounds in increasing the resistance of capillaries to rupture[1]. The term "vitamin P" has been applied to flavonoids having this property; "bio-

flavonoid" has been suggested as being more appropriate[2]. Recently the administration of rutin, a member of this class of plant pigments, has been reported to increase the survival rate of dogs submitted to total body irradiation of approximately mid-lethal doses of x-rays[3]. Progress in understanding these effects is dependent on the development of satisfactory analytical methods for the flavonoid pigments.

Flavone, the parent compound of the hydroxyflavones with which the present research is concerned, is 2-phenylbenzo-$\gamma$-pyrone (See Figure 1). Flavones in which a hydroxyl group occurs in the 3-position are called flavonols. If the double bond between positions two and three is saturated, the compound is termed a flavanone. The hydroxyl groups in naturally occurring flavone-type compounds may be found free, methylated, or in glycosidic combination with various sugars. The most usual sugars are glucose, rhamnose, and rutinose.

A part of the research reported here was directed toward the extension of the paper chromatographic method for separating mixtures of the flavones and for their qualitative analysis. It was the purpose of this phase of the work to investigate several new solvent systems for the separation of these compounds, and to establish standard $R_f$ values in the several solvent systems for a number of the pigments. A new color reaction for locating the flavones on developed chromatograms is reported.

A second portion of the research was directed toward the development of methods for the isolation of the pigments involving the use of new chromatographic media such as starch and cellulose derivatives.

A third section of this thesis describes the application of chromatographic procedures to the characterizarion of flavonoid compounds

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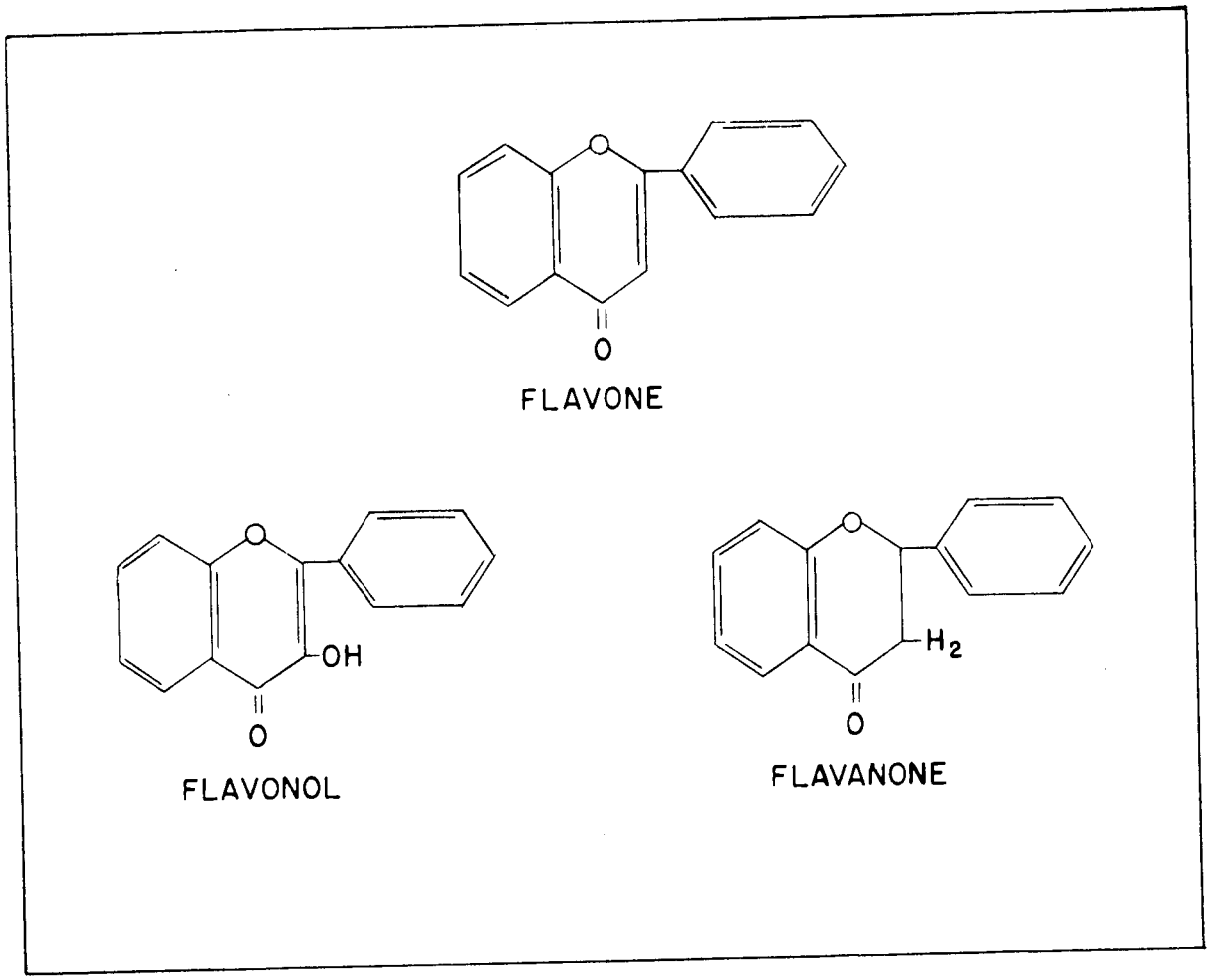



Figure 1

Structures of Some Flavonoid Compounds

in certain natural products. Among the sources of flavonoid pigments studied were Big Bend locoweed, Tokay grapes, and Thompson White Seedless grapes. Ion-exchange resin chromatography was applied in these studies.

Since the identification of any organic compound depends ultimately upon the preparation of characteristic derivatives, a fourth important portion of the research was devoted to the attempt to prepare new derivatives of the flavone family. An investigation of the use of flavanone 2,4-dinitrophenylhydrazones for crystallographic identification of the parent compounds has been made.

A fifth part of the research has been directed toward the preparation of carbon-14 labeled flavone derivatives. Such studies, in addition to clarification of the chemistry of these compounds, should lead to the use of radioactive derivatives in the study of their metabolism and action in plant and animal systems. In connection with these studies, some of the reactions involved in the preparation of this class of compounds were investigated from the standpoint of their reversibility. The exchange of radioactive moieties for non-radioactive ones in intermediates leading to these compounds has led to a unique method of preparation of carbon-14 labeled flavonoids. Attempts were made to exchange radioactive acyl groups into aromatic ketones under the conditions of the Friedel-Craft acylation reaction and to exchange the radioactive benzal group into compounds formed by carbonyl-methylene condensation reactions.

# CHAPTER II

# HISTORICAL

## Chemistry of Flavonoid Compounds

When water solutions of the flavonoid pigments are treated with magnesium and concentrated hydrochloric acid, some shade of red develops. Geismann and Clinton[4] have studied the products formed by this reaction and have found them to be predominantly 4-hydroxyflavanes. Treatment of the flavones with either lead acetate or basic lead acetate produces lead salts which are usually yellow. Silver nitrate is reduced by these compounds. Aqueous solutions of the pigments give deep yellow colors when treated with concentrated sulfuric acid or ammonium hydroxide. Highly fluorescent yellow compounds result when the flavones are treated with aluminum or thorium salts. The pigments are strongly adsorbed on aluminum hydroxide. This property affords a possible method for separating the pigments from certain impurities. Various shades of green are produced by treatment of the flavonoid compounds with alcoholic ferric chloride.

Table I shows the chemical names of the flavonoid compounds which were studied in this research.

## Paper Chromatography of Flavonoid Pigments

With the development of the paper chromatographic methods of analysis of Consden, Gordon, and Martin[5] it became possible to separate complex mixtures of substances occurring in nature in extremely small quantities. Bate-Smith[6] and Wender and Gage[7] first applied this method to the analysis for flavone-type compounds. In a subsequent study, Bate-

TABLE I

Chemical Names of Flavonoid Compounds

Flavonol Aglycones

Gossypetin - 3,3',4',5,7,8 -hexahydroxyflavone

Kaempferol - 3,4',5,7 - tetrahydroxyflavone

Morin - 2',3,4',5,7 - pentahydroxyflavone

Nortangeretin - 3,4',5,6,7 - pentahydroxyflavone

Patuletin - 3,3',4',5,7 - pentahydroxy-6-methoxyflavone

Quercetagetin- 3,3',4',5,6,7 - hexahydroxyflavone

Quercetin - 3,3',4',5,7 - pentahydroxyflavone

Rhamnetin - 3,3',4',5 pentahydroxy-7-methoxyflavone

Robinetin - 3,3',4',5',7 pentahydroxyflavone

Flavonol Glycosides

Gossypetin - 8-glucoside of gossypetin

Gossypitrin - 7-glucoside of gossypetin

Isoquercitrin - 3-glucoside of quercetin

Quercemeritrin - 7-glucoside of quercetin

Quercitrin - 3-rhamnoside of quercetin

Robinin-3-robinoside of kaemferol

Rutin - 3-rutinoside of quercetin

Xanthorhamnin - 3-trirhamnoside of rhamnetin

Table I (Continued)

Flavone Aglycones

Acacetin - 5,7-dihydroxy-4-methoxyflavone

Apigenin - 4',5,7-trihydroxyflavone

Auranetin - a pentamethoxyflavone [8]

Chrysin - 5,7-dihydroxyflavone

Genkwanin - 4',5-dihydroxy-7-methoxyflavone

Isowogonin - 5,8-dihydroxy-7-methoxyflavone

Norwogonin - 5,7,8-trihydroxyflavone

Oroxylin A - 5,7-dihydroxy-6-methoxyflavone

Wogonin - 5,7-dihydroxy-8-methoxyflavone

Flavanone Aglycones

Butin - 3',4',7-trihydroxyflavanone

Hesperitin - 3',5,7-trihydroxyflavanone

Homoeriodictyol - 4',5,7-trihydroxy-3'-methoxyflavanone

Liquiritiginin - 4',7-dihydroxyflavanone

Flavanone Glycosides

Hesperidin - 7-rhamnoside of hesperitin

Narigin - 7-rhamnoglucoside of 4',5,7-trihydroxyflavone

Neohesperidin - A rhamnosidoglucoside of hesperitin [9]

Chalcones

"Hesperidin methyl chalcone" - a product obtained from the methylation of hesperidin chalcone [10]

Phloretin - β-(p-hydroxyphenyl) phloropropiophenone

Related Compounds

Esculetin - 6,7-dihydroxycoumarin

Pomiferin - 5-hydroxy-3-(3,4,-dihydroxyphenyl)-8,8-dimethyl-6-(3-methyl-2-butenyl)-4,8-benzo(1,2-b; 3,4-b')dipyran-4-one. [11]

Smith and Westall[12] have shown that an approximately linear relationship exists between the number of substituent hydroxyl groups in a flavone nucleus and its rate of migration in a given solvent system. The paper chromatographic method also has been extended to include a quantitative assay for certain of the pigments[13].

## Chromatographic Isolation Procedures

Heretofore the isolation of flavonoids from their natural sources have been achieved by laborious extraction and precipitation techniques.

Gage, Gallemore, and Wender[14] have made a study of the conventional chromatographic adsorbents for use in isolations. They found that the usual adsorbents were not well suited for use with flavone compounds, since some bound the pigments too tightly and others did not adsorb them at all. Decomposition of the pigments was caused by certain of the adsorbents.

Poole and co-workers[15] have reported the use of potato starch columns in the isolation of rutin from tomatine concentrates.

Very recently Morris, Gage, Detty, and Wender[16] have reported the successful use of the cation-exchange resin Amberlite IRC-50 in the isolation of flavonoid compounds. This technique, developed after the studies reported herein were completed, appears to be an excellent one.

## Application of Chromatographic Procedures to the Characterization of Naturally Occurring Flavones

While investigating the toxic principle of Big Bend locoweed, *Astragalus earlei*, Chervenka[17] noted that a bright yellow substance present in extracts of the weed was adsorbed on Amberlite IRC-50

cation exchange resin. There have been no previous reports in the literature concerning the flavonoid constituents of any Astragalus species.

In an examination of the "vitamin P potency" of several foodstuffs Scarborough[18] has found that "white and black" grapes were very effective in increasing the capillary resistance of cats, rats, guinea pigs, and humans. The increase in capillary resistance was measured by noting the vacuum necessary to produce a hemorrhagic area of a given size.

It was decided to investigate the flavonoid constituents of locoweed and of grapes in order to test the practicality of using paper chromatography as a tool in qualitative analysis for the flavonoid compounds present in natural sources and, in the case of the grapes, to determine the nature of the flavonoids present in connection with the "vitamin P" problem.

## Preparation of Flavonoid Derivatives

The derivatives which are usually prepared in the characterization of flavonoid compounds are the oximes and, in the hydroxylated compounds, the acetates, benzoates, and methyl and ethyl ethers. Several of the hydrobromides, hydroiodides, and metallic salts of the phenolic compounds have been reported[19]. These are, however, of limited usefulness.

Mozingo and Adkins[20], in a study of the catalytic hydrogenation of pyrone derivatives, prepared the 2,4-dinitrophenylhydrazones of flavanone and flavone as derivatives for characterization.

## Isotopic Exchange Reactions

Brief Survey - An exchange reaction is a reaction in which an atom or a radical present in a given compound is replaced by an atom or radical present in the environment of the compound. At the present time the only method of detecting an exchange reaction in which atoms or radicals identical with those originally present in the compound are exchanged is to use an isotope of the element being exchanged.

One of the earliest examples of an exchange reaction was reported by Hevesy in 1920.[21] A solution of lead chloride was treated with lead nitrate which contained some thorium B (lead-212). The lead chloride was isolated and purified and was found to contain some of the lead-212. By the same method it was found that exchange occurred between plumbous and plumbic ions in a solution of their acetates.[22]

Since stable and radioactive isotopes have been available for widespread use, a large number of exchange reactions have been investigated. The exchange reactions of the radiohalogens have been studied under a wide variety of conditions. As an example, Clusius and Haimer [23] have reported that the chlorine-35 atoms in hydrogen chloride exchange with arsenious chloride, phosphorous trichloride, phosphorous oxychloride, silicon tetrachloride, sulfur monochloride, and solid potassium chloride. Hydrogen bromide containing a radioactive bromine atom was found to exchange rapidly with gaseous bromine.[24] Alkyl bromides have been found to exchange with gaseous radiobromine and radioactive hydrogen bromide [25]. The chloride ion exchanges rapidly with chlorine gas in aqueous solution [26]. A great many other exchange reactions of the halogens are reported.

The exchange reactions of ions in different oxidation states have been studied. Daudel, Daudel, and Martin[27] have reported exchange between ferrous and ferric, cuprous and cupric, thallous and thallic, and mercurous and mercuric ions. The exchange of cobaltous and cobaltic ions is reported to be rapid, while the exchange of these ions with their hexammino complexes proceeds very slowly[28]. It has been shown that an exchange occurs between metallic zinc and zinc ions at pH 5, before an oxide film coats the metal[29]. The cupric ion has been found to exchange with metallic copper[30].

Sulfur-35 has been used to study various exchange reactions involving this element. Libby[31] has shown that sulfide and thiosulfate ions exchange. Edwards and co-workers found that elemental sulfur-35 exchanged with carbon disulfide[32].

Exchange reactions between the isotopes of hydrogen have been studied for many years and are so well known and numerous that only a few examples need be given.

Acid hydrogen, i. e., hydrogen atoms which are more or less ionic in character can generally be replaced by deuterium by treatment with heavy water. Hydrogen atoms directly linked with carbon in aliphatic and aromatic hydrocarbons in general withstand exchange by deuterium. This does not hold for hydrogen atoms activated by adjacent negative groups, however. Phenols exchange the hydrogen atoms in the positions in which ordinary substitution reactions occur, i. e. the 2, 4, and 6 positions. By the action of certain catalysts or heat, more firmly bound hydrogen atoms of organic compounds may be induced to exchange with isotopic hydrogen[33].

Several reports concerning the exchange of carbon isotopes have appeared recently. Long[34] has found no exchange between the oxalate and the chromioxalate ion during the racemization of the latter. In a later paper, Long[35] reported that ferric and aluminum trioxalate ions exchanged rapidly with labeled oxalate ions, while the cobalt trioxalate ion did not. Ruben[36] and co-workers have noted a rapid exchange reaction between sodium acetate and acetic anhydride at room temperature. Brandner and Urey[37], using the stable isotope, carbon-13, observed a surface-catalyzed exchange between carbon monoxide and carbon dioxide. The exchange of carbon dioxide between barium carbonate and the atmosphere has been reported[38,39]. Calvalieri and Brown have found an exchange between a formamido group and formamide.[40] The carbonate ion has been shown to exchange with the carbonatotetrammine cobaltic ion[41]. Tsai and Kamen found no exchange of cyano groups between cyanide ions and nitriles[42]. Grant and Turner[43] observed an exchange reaction between sodium formate and sodium bicarbonate when the two were heated together under pressure.

Use in Kinetic Studies - The information which is gained from studies of exchange reactions is of much theoretical and practical importance. Exchange techniques afford a useful tool for the investigation of the kinetics of certain reactions. Wilson and Dickinson[44] were able to calculate the specific rate constants for the oxidation of trivalent arsenic to pentavalent arsenic by iodide ions and for the reverse of this reaction, the reduction of pentavalent arsenic to trivalent arsenic by iodine, by measuring the rate of exchange of the two forms of arsenic in the presence of iodide ion and free iodine.

Use in Structure Determination - Exchange reactions have furnished some valuable information in the field of molecular structure. Voge[45] has shown that one of the sulfur atoms of the thiosulfate ion exchanges rapidly with the sulfite ion at 100° while the other atom exchanges much more slowly. The conclusion which is drawn is that the two sulfur atoms are not equivalent. In the work of Long[35] it was found that chromium and cobalt ions did not exchange with their trioxalate complexes while ferric and aluminum ions did. This is interpreted as meaning that the ferric and aluminum ions are bound to the complexes by ionic bonds while chromium and cobalt are bound by covalent bonds.

Recently the lack of exchange between labeled durohydroquinone and duroquinone in the formation and decomposition of the molecular compound duroquinhydrone has been demonstrated[46]. This information indicates that duroquinhydrone does not exist as a symmetrical resonance hybrid structure, as previously postulated.

Use in Determination of Reaction Mechanisms - Reaction mechanisms have been investigated by studying rates of exchange reactions. Thomas[47] proposed that the racemization of the chromioxalate ion, $Cr(C_2O_4)_3^{\equiv}$ involved an ionization of the ion as the rate determining step.

$$\text{d or l} \quad Cr(C_2O_4)_3^{\equiv} \rightleftharpoons Cr(C_2O_4)_2^{=} + C_2O_4^{=}$$

Long[34] has been able to test this hypothesis by comparing the rates of racemization and of exchange of radioactive oxalate ion with the complex. That the rates were different indicated that the ionization step was not rate-determining.

Use as Test for Reversibility - One of the most obvious and useful applications of exchange reactions is the test for the reversibility of a reaction. If a dynamic equilibrium exists between the reactants and products of a reaction, then, if one of the reactants which contains a radioactive atom is mixed with one of the products under suitable conditions and allowed to stand until exchange is complete, the product on isolation will contain a definite calculable fraction of the radioactivity originally contained in the reactant.

Use in Synthesis - Exchange reactions have been used as methods for preparing certain labeled compounds. Isotopic hydrogen may be conveniently introduced into certain positions of molecules by this method. Kamen[48] has suggested that exchange could be used for introducing isotopic carbon atoms into organic compounds.

Use in Isotope Enrichment - Certain heterogeneous exchange reactions have been used in the fractionation of isotopes. For example, carbon-13 may be separated from carbon-12 by an exchange procedure.[49]

Theory of Exchange Reactions - McKay[50] has derived the rate equation for simple homogeneous exchange reactions. Consider a reaction between the molecular species A and B in which atoms of a given element X, common to both species, are exchanged under a specific set of conditions at a constant rate. In this discussion, the labeled atom of X will be assumed to be radioactive for ease of presentation.

Let R = the constant rate of exchange of X atoms between A and B (gram-atoms per liter)

$t$ = time

$(A)$ = concentration of X (active plus inactive) in the A form (gram atoms of X per liter)

$(B)$ = concentration of X (active plus inactive) in the B form (gram atoms of X per liter)

$(A')$ = concentration of radioactive X in the A form at time t (gram atoms of X per liter)

$(B')$ = concentration of radioactive X in the B form at time t (gram atoms of X per liter)

$S_A$ = fraction of X atoms in A that are active

$S_B$ = fraction of X atoms in B that are active

$F$ = fraction of exchange.

The fraction of exchange may be generally defined as

$$F = \frac{S_A - S_{A_o}}{S_{A_\infty} - S_{A_o}} = \frac{S_B - S_{B_o}}{S_{B_\infty} - S_{B_o}} \tag{1}$$

where subscripts o and $\infty$ refer to $t = 0$ and $t = \infty$, respectively. If A were initially inactive, then

$$S_{A_o} = 0 \tag{2}$$

and

$$F = \frac{S_A}{S_{A_\infty}} \tag{3}$$

Since $S_A = \frac{(A')}{(A)}$ and $S_B = \frac{(B')}{(B)}$ then

$$F = \frac{(A') - (A_o')}{(A_\infty') - (A_o')} = \frac{(B') - (B_o')}{(B_\infty') - (B_o')} \tag{4}$$

The rate of increase of the concentration of radioactive X in the A form is its rate of formation decreased by its rate of decomposition. This is expressed by the equation,

$$\frac{D(A')}{dt} = RS_B\left[1-S_A\right] - RS_A\left[1-S_B\right] = R\left[S_B - S_A\right] \tag{5}$$

$$= \frac{R}{(A)(B)}\left[(A)(B') - (B)(A')\right] \tag{6}$$

Assuming no radioactive decay,

$$(A') + (B') = (A_\infty') + (B_\infty')$$

$$\frac{(B_\infty')}{(A_\infty')} = \frac{(B)}{(A)} \tag{7}$$

If (B') in equation 5 is eliminated by substitution from equations 6 and 7,

$$\frac{d(A')}{dt} = \frac{R}{(A)(B)}\left[(A)(A_\infty') + (B)(A_\infty') - (A)(A') - (B)(A')\right]$$

$$= \frac{R}{(A)(B)}\left[(A) + (B)\right]\left[(A_\infty') - (A')\right]$$

and

$$\frac{d(A')}{(A_\infty') - (A')} = \frac{R\left[(A) - (B)\right]}{(A)\ (B)}\,dt \tag{8}$$

Integration of equation 8 gives

$$-\ln \quad (A'_{\infty})-(A') \quad = \quad \frac{R \quad [(A) - (B)]}{(A) \quad (B)} t + \text{constant} \qquad (9)$$

The constant of integration may be evaluated at $t = 0$ when $(A') = (A_o')$

$$\text{Constant} = -\ln \quad (A'_{\infty})-(A_o') \qquad (10)$$

Substitution of equation 10 in equation 9, and rearrangement, gives

$$Rt = \frac{(A)(B)}{(A) - (B)} \ln \quad \frac{[(A_{\infty}')-(A_o')] - [(A')-(A'_o)]}{(A_{\infty}') - (A'_o)}$$

and

$$Rt = - \frac{(A)(B)}{(A)-(B)} \ln \quad (1-F) \qquad (11)$$

Rearrangement of equation (11) gives

$$F = 1-e^{\frac{(A) - (B)}{(A)(B)} Rt} = \frac{(A') - (A'_o)}{(A_{\infty}')-(A'_o)} \qquad (12)$$

Rearrangement of equation 12 yields

$$(A') = \left[(A'_{\infty}) - (A'_o)\right] \quad \left[1-e^{-\frac{-(A) + (B)}{(A) \quad (B)} Rt}\right] + (A'_o) \qquad (13)$$

Since R, (A), (B), $(A'_o)$ and $(A'_{\infty})$ are all constant for a given experiment, the differential form of equation 13 may be written

$$\frac{d(A')}{dt} = k_1 e^{-k_2 t} , \qquad (14)$$

where $k_1$ and $k_2$ are constants.

It may be seen from equation 14 that the rate of appearance of radioactivity in the A form follows a simple exponential law.

In experiments described in this thesis it has been necessary to evaluate the quantity $A'_{\infty}$ when A is initially inactive. When $t = \infty$

$$(B'_{\infty}) = (B'_{o})-(A'_{\infty}) \qquad (15)$$

Substituting this value into equation 7 gives

$$(B'_{o})-(A'_{\infty}) = \frac{(B)}{(A)} (A'_{\infty}) \qquad (16)$$

Simplification and rearrangement of this expression yields

$$(A'_{\infty}) = \frac{(B'_{o})}{1-\frac{(B)}{(A)}} \qquad (17)$$

Thus, knowing the amount of radioactivity originally present in form B and the molar proportions of A and B it is possible to calculate the amount of radioactivity which will be present in form A when exchange is complete.

## Friedel-Crafts Acylation Reaction

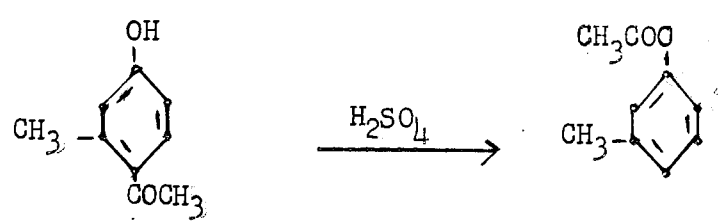
It is known that the Friedel-Crafts alkylation reaction is reversible. For example Boedtker and Halse[51] have shown that when polyalkyl benzenes were heated with benzene and aluminum chloride, monoalkyl derivatives of benzene resulted. Conversely, from the treatment of ethylbenzene with aluminum chloride, diethylbenzene and benzene may be isolated.[51] A large number of such reactions are known. The Jacobson reaction in which reorientation of alkyl groups on aromatic nuclei under the influence of strong acid catalysis is one manifestation of this reversibility.

Unlike the alkylation reactions, the Friedel-Crafts acylation is usually considered to be irreversible in the absence of proof to the contrary. Olivier[52] looked specifically for evidence of reversibility in the formation of benzophenone. The experiment consisted of preparing an unspecified quantity of a 0.2 molar solution of the benzophenone-aluminum chloride complex in benzene, saturating the solution with hydrogen chloride, allowing the mixture to stand for five days at 30° and analyzing for benzoyl chloride. The fact that no benzoyl chloride was found was evidence for the irreversibility of the reaction. It is unfortunate that such a large quantity of benzene was used in the experiment since, if one assumes reversibility of the reaction according to the equation,

$$C_6H_5COC_6H_5 + HCl \underset{}{\overset{AlCl_3}{\rightleftharpoons}} C_6H_5COCl + C_6H_6$$

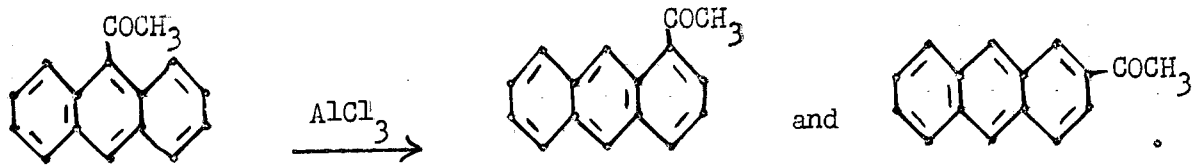
the excess of benzene should force the reaction toward the left according to the law of mass action. Under these conditions, the amount of benzoyl chloride formed would be expected to be very small.

A few examples are known which seem to involve the migration of an acyl group by rupture of a carbon-carbon bond. Rosenmund and Schnurr[53] found that p-hydroxy ketones having an alkyl group ortho to the acyl group are converted to m-alkylphenyl esters on heating with certain acids:



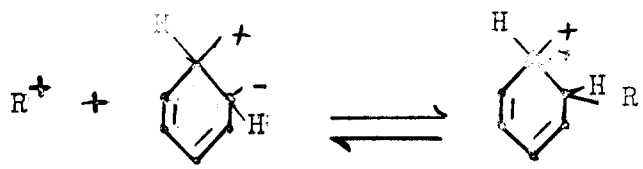
They also observed that when the p-hydroxy ketone shown above is heated with aluminum chloride it is converted to the isomeric o-hydroxyketone.

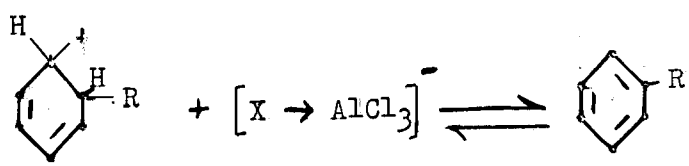
When meso-anthracyl alkyl ketones are treated with aluminum chloride at an elevated temperature or in high concentration, they are converted to $\alpha$- and $\beta$-anthracyl alkyl ketones:[54]




The mechanism which is now generally accepted for the Friedel-Craft alkylation involves the intermediate formation of the carbonium ion and the ion $AlCl_4^-$, followed by attack of the positive ion on the aromatic nucleus:

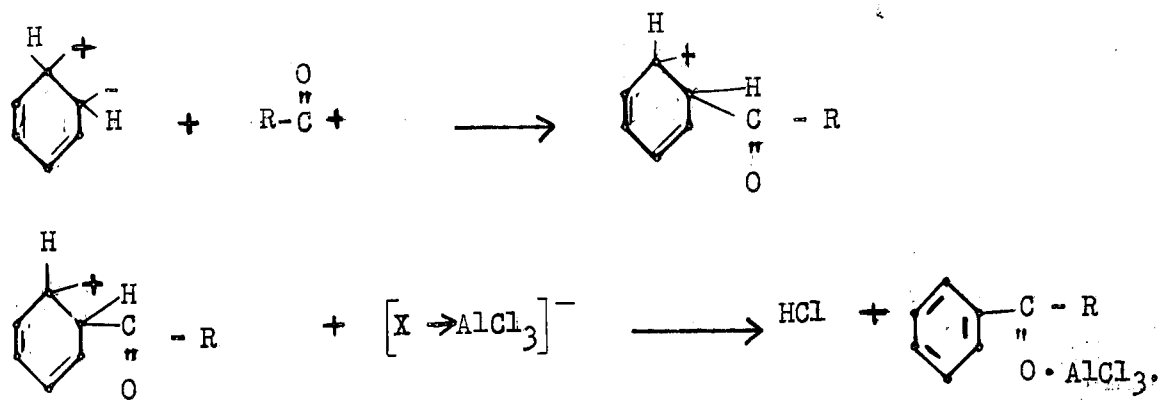
$$R\text{-}X + AlCl_3 \rightleftharpoons [R\text{-}X \rightarrow AlCl_3] \rightleftharpoons R^+ + [X \rightarrow AlCl_3]^-$$




A similar mechanism is written for the acylation reaction.

$$R - \overset{O}{\overset{\|}{C}} - X + AlCl_3 \rightleftharpoons R - \overset{O}{\overset{\|}{C}}^{+} + [X \rightarrow AlCl_3]^-$$



Evidence for an ionic mechanism of the alkylation reaction has been found in conductance studies of Wertyporoch and coworkers [55,56]. Evidence for the formation of an ion of the type $AlCl_4^-$ has been supplied by Fairbrother [57], who found that when the reactions were carried out with aluminum chloride containing radioactive chlorine, the radioactivity found in the hydrogen chloride was one-fourth the amount originally present in the aluminum chloride. Similarly, a reaction carried out with an alkyl bromide and aluminum chloride gave a proportion of hydrogen bromide to hydrogen chloride of 1:4 [58].

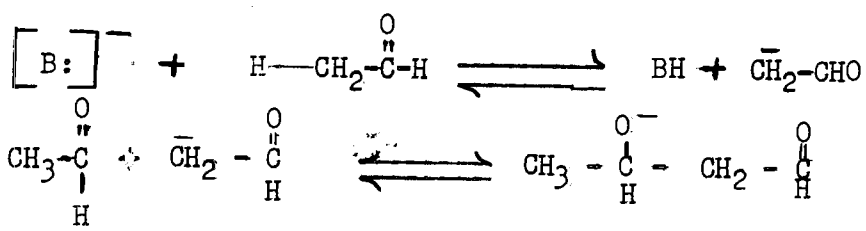
There are however, significant differences between the alkylation and acylation reactions. The alkylation reaction requires only a small amount of the catalyst, whereas the acylation reaction requires at least a mole of the catalyst for each mole of product formed. This is explained on the basis that a stable complex is formed between the ketone, resulting from the acylation reaction, and aluminum chloride, thus removing the aluminum chloride from reaction.

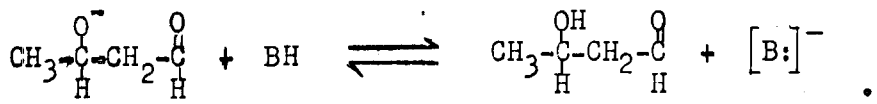
## Carbonyl-Methylene Type Condensations

Reactions which involve the condensation of the carbonyl group of one molecule with the active methylene group of another to form a carbon-carbon bond constitute a very general transformation. Since such reactions may be carried out under a wide variety of catalytic conditions, both acid and basic in nature, further classification of these condensations has usually been made on the basis of the catalyst used. Unfortunately this classification is confused by the tendency of authors to catalogue the types as "name" reactions, e. g. Claisen, Knoevenagel, etc. Since there is not general agreement in this effort, the classification used by one author, Alexander[59], will be utilized in the discussion to follow. The extent of reversibility of the various reactions of this type is not too well defined. Excellent evidence is available in some cases; in others there are few data.

Several of the base-catalyzed carbonyl-methylene types of condensation reactions appear to have similar mechanisms. All appear to be best classified on the basis of a carbanion intermediate.

Aldol Condensation - A mechanism of the aldol condensation was originally suggested by Lapworth[60] in 1904. Watson[61] suggests that the essentials of Lapworth's mechanism have changed little and were essentially correct. The mechanism may be written as follows:

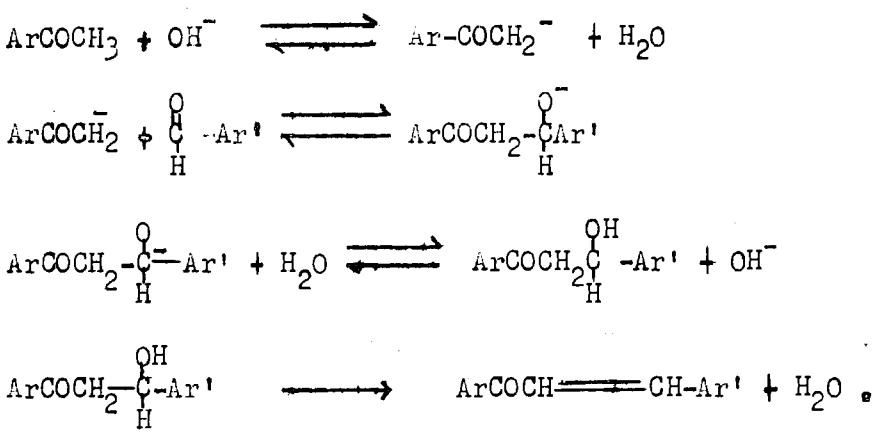




It is generally conceded[61,62] that the reaction is reversible through the step in which the aldol is formed. If the reaction is carried from this point through a dehydration, however, it is assumed to be irreversible[63,64].

Bell[65] has studied the kinetics of condensation of acetaldehyde to aldol and has found that the reaction is of the first-order with respect to acetaldehyde.

<u>Claisen-Schmidt Reaction</u> - The catalyst for the reaction is 10% aqueous sodium hydroxide. Its mechanism, as suggested by Alexander[66] is as follows:



This reaction, like the aldol condensation, is usually considered to proceed in reversible steps to the final irreversible dehydration. Gettler and Hammet[64] have studied the kinetics of the reaction between benzaldehyde and acetone and methyl ethyl ketone. They have found that the reactions are first-order with respect to both the aldehyde and ketone and that the rate constants bear a linear relationship to the square root

of the base concentration. These workers suggest that the reactions are not reversible. Coombs and Evans[67] have found that the reaction between acetophenone and benzaldehyde is kinetically bimolecular. The rate was observed to be proportional to the base concentration. Bonhoeffer and Walters[63] have found that no deuterium was taken up from heavy water when acetaldehyde was condensed to form aldol. Chelintzev and Nikitin[68] have found that furfural can be displaced from its union with acetone by aliphatic aldehydes but not by aromatic aldehydes. They consider that this reaction proceeds by a hydration of the double bond followed by a reversal of the aldol reaction and finally reaction between the replacing aldehyde and acetone[69].

<u>Knoevenagel Reaction</u> - While no classical Knoevenagel condensation reactions have been studied here, one reaction similar to this condensation is reported, i. e., the reaction of benzaldehyde with 2,4-dinitrotoluene in the presence of piperidine. Cope[70] has studied the mechanism of the Knoevenagel reaction between ethyl cyanoacetate and methyl n-amyl ketone. He concludes that the reaction proceeds as follows:

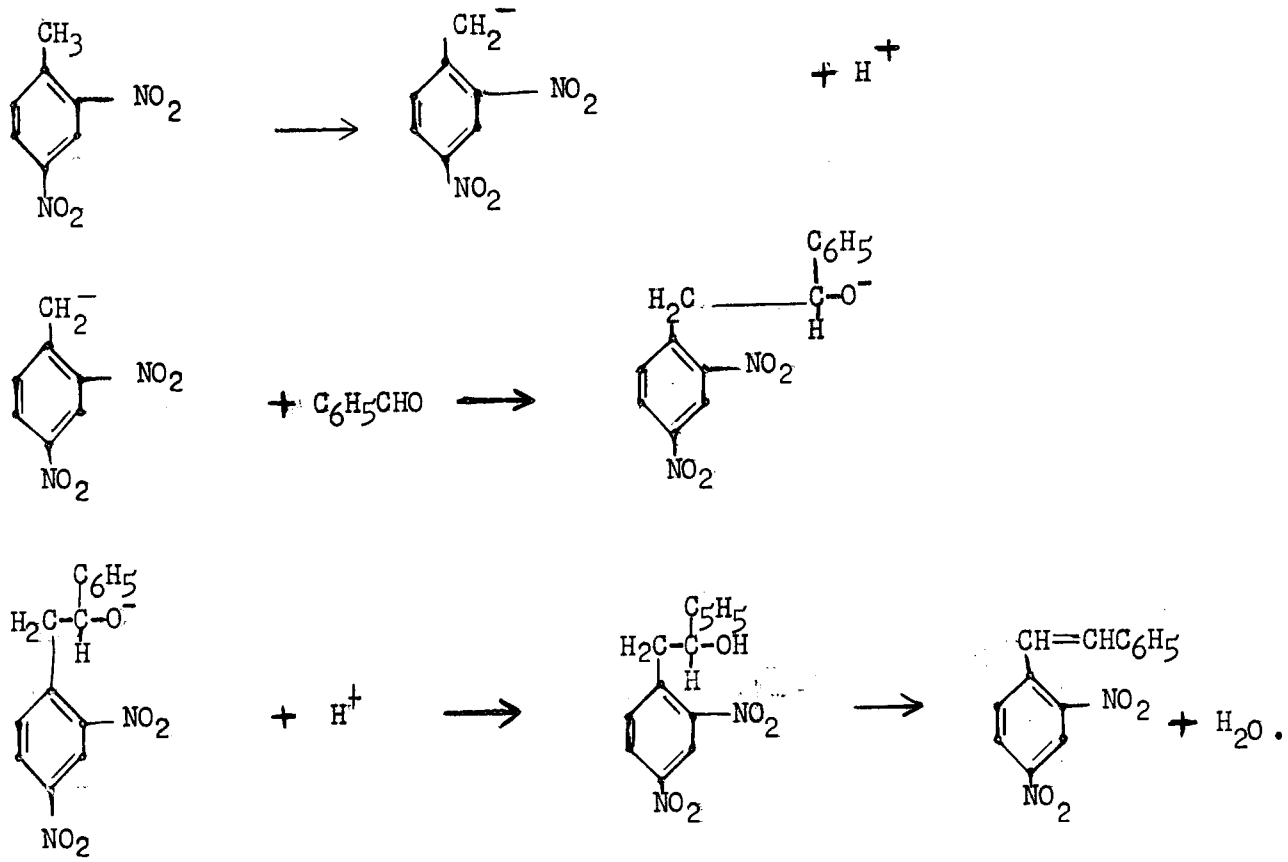
$$CH_2(CN)COOC_2H_5 \rightleftharpoons H^+ + \bar{C}H(CN)COOC_2H_5$$

$$C_5H_{11}-\overset{O}{\overset{\|}{C}}-CH_3 + \bar{C}H(CN)COOC_2H_5 + H^+ \rightleftharpoons$$

$$C_5H_{11}-\underset{OH}{\overset{CH_3}{C}}-CH(CN)COOC_2H_5 \rightleftharpoons H_2O + C_5H_{11}-\overset{CH_3}{C}=\overset{CN}{\underset{COOC_2H_5}{C}}$$

The complete reversibility of the reaction was supported by the fact that when the unsaturated compound was heated with water, some of the ketone was recovered.

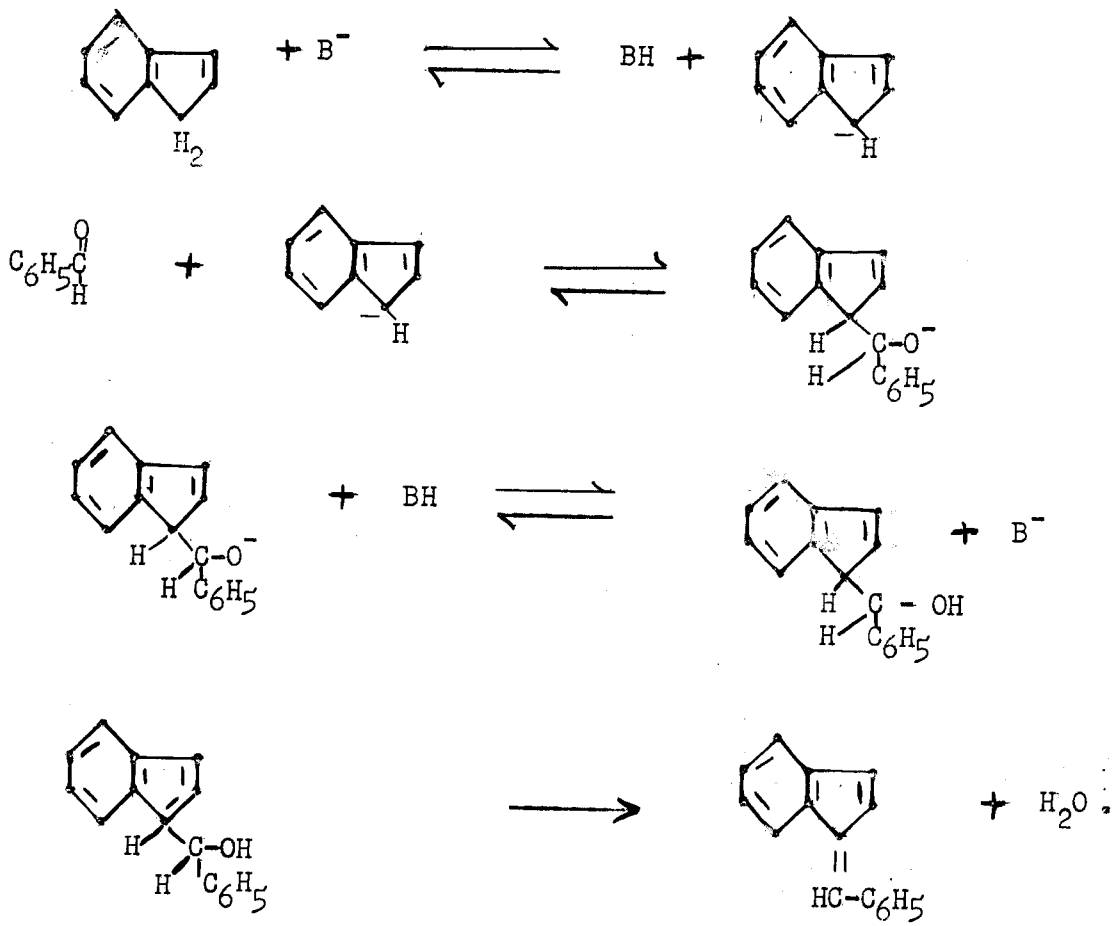
Remick[71] proposes a mechanism for the condensation of benzaldehyde and 2,4-dinitrotoluene. It is essentially the same as that offered for other condensations of this type.



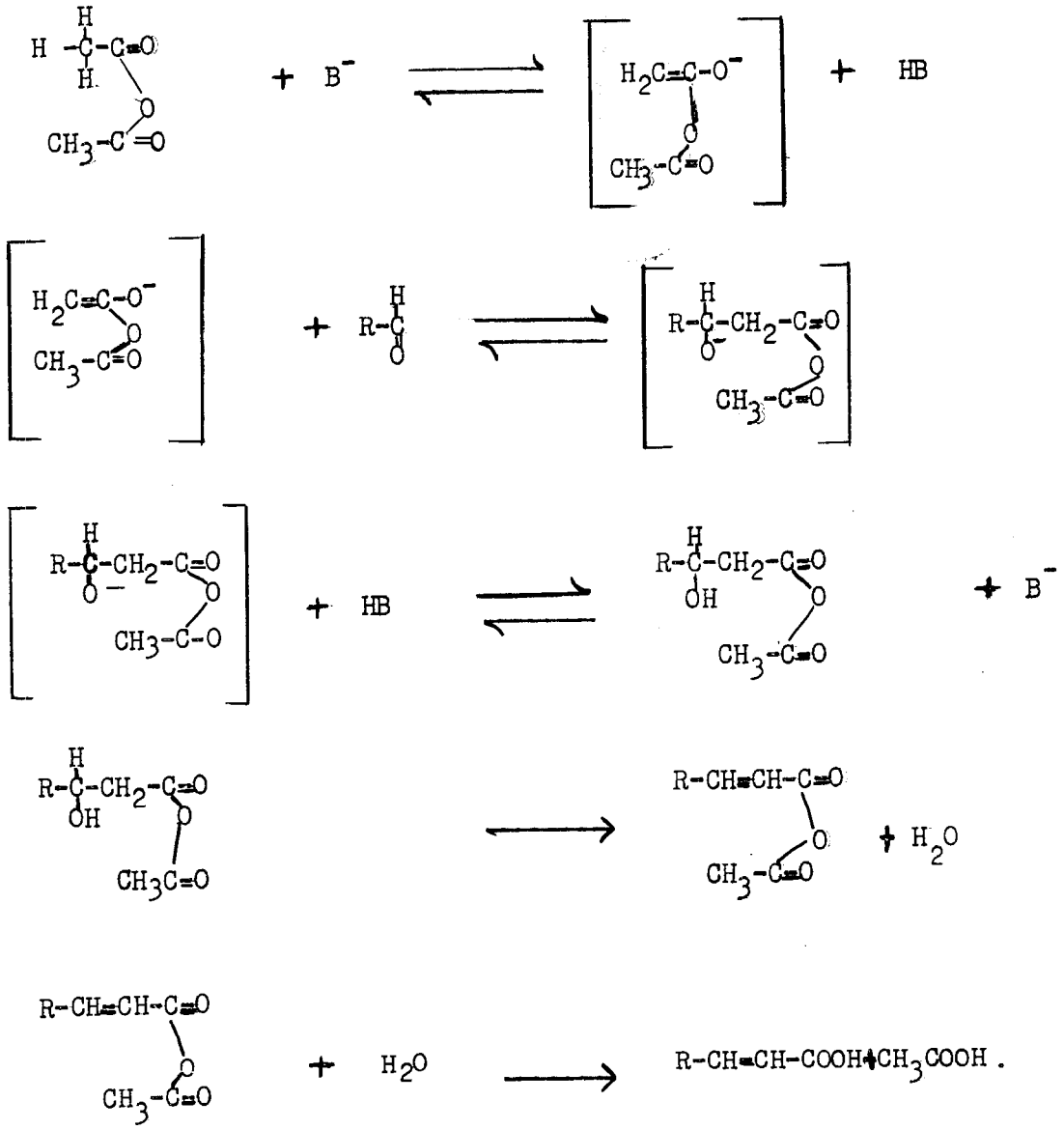
No mention is made of the reversibility of the reaction.

The reaction between benzaldehyde and indene to give 8-phenylbenzofulvene may conveniently be considered to be a type of the Knoevenagel reaction. The preparation of 8-phenylbenzofulvene is carried out in methanolic potassium hydroxide[72].

The reaction probably proceeds as follows:




Perkin Reaction - The Perkin reaction is similar to the reactions discussed above. In this condensation between an aromatic aldehyde and acetic anhydride, the usual catalyst is sodium acetate. Hauser and Breslow [73,74] have proposed the following mechanism for the Perkin synthesis:



Hauser considers the Perkin reaction to be irreversible.

<u>Claisen Reaction</u> - The Claisen condensation is similar to the reactions studied here. Dieckmann[75] found that ethyl α-propionylpropionate, formed by the self-condensation of ethyl propionate, reverted to ethyl propionate on treatment with alcoholic sodium ethoxide. Hauser and Hudson[76]

observed that ethyl benzoyldimethylacetate reverted to ethyl benzoate and ethyl isobutyrate when allowed to stand in the presence of sodium ethoxide and triphenylmethane. Both of these compounds are by-products of the condensation of the two esters in the presence of triphenylmethyl sodium. The mechanism of this reaction is similar to all those discussed in this section, the first step being the formation of a carbanion followed by addition of the carboxyl-carbon of the ester and elimination of the alkoxide ion.

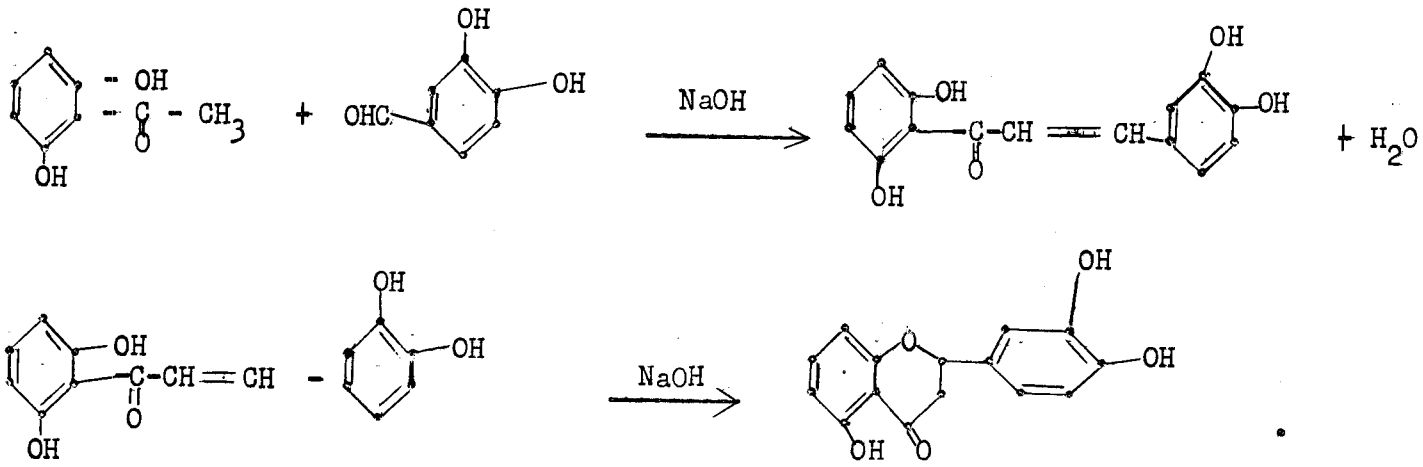
## Synthesis of Labeled Flavanones Utilizing Isotopic Exchange Reactions

Although the preparation of carbon-14 labeled flavonoid compounds has not been reported, the chemistry of the preparation and interconversions of the unlabeled compounds is well understood[77].

Flavanones serve as the starting materials for the preparation of the flavones and the flavonols. Dehydrogenation with selenium dioxide or phosphorous pentachloride yields the corresponding flavone. Flavonols are produced by treatment of the flavanones with amyl nitrite to give 3-isonitroso derivatives, and subsequent hydrolysis.

Preparation of polyhydroxybenzalacetophenones, for conversion to the isomeric polyhydroxyflavanones, has usually involved condensation of the appropriately substituted benzaldehyde with the desired o-hydroxyacetophenone with a basic catalyst[78]. For instance, 3',4',5-tri-

hydroxyflavanone may be prepared by the following series of reactions:




Although the benzaldehyde—acetophenone condensations are quite efficient in some cases, the yields of the more highly hydroxylated benzalacetophenones from the condensation reaction are frequently low. As examples, Balaiah, Row, and Seshadri[79] report that 2',4', 5' - trihydroxy-2,6'-dimethoxybenzalacetophenone is formed in 43% yield, 2'-hydroxy-3,4,5',6' tetramethoxybenzalacetophenone in 38% yield, and 2'-hydroxy-4,5',6'-trimethoxybenzalacetophenone in 27% yield. Procedures giving such yields are therefore not attractive as preparative methods for radioactive flavonoid compounds.

The isomerization of 2'-hydroxybenzalacetophenone to flavanone is reported[80] to give a quantitative yield. Ring-closure reactions of polyhydroxy benzalacetophenones produce good yields of the corresponding flavanones.

# CHAPTER III

# EXPERIMENTAL

## Studies on Naturally-Occurring Flavonoid Compounds

### Paper Chromatography of Flavonoid Pigments

One-Dimensional Descending Paper Chromatography - Strips for one-dimensional, descending paper chromatography were prepared by cutting sheets of Whatman No. 1 filter paper into strips 2.5 x 57 cm. Reagent-quality solvents and distilled water were used in all cases. After mixing, the two-phase systems were allowed to stand in separatory funnels until complete separation was achieved. The homogeneous solvents were thoroughly mixed and used immediately. A "Chromatocab" chamber, (University Apparatus Company, Berkeley, California) was used for the chromatography. The solvents were placed in the chamber troughs and allowed to stand several hours before use. The compound to be chromatographed was dissolved in ethanol in a concentration of 1 mg. per ml. Samples containing 0.020 to 0.040 mg. were spotted on the paper strips 8 cm. from one end. The ethanol was evaporated rapidly enough to keep the spot approximately 1 cm. in diameter. The end of the strips nearest the sample spots were suspended in the troughs of the chamber.

After the solvent had moved about 30 cm., the strip was removed from the chamber and allowed to dry. Examination in ultraviolet light usually revealed the pigment zone as a yellow or brown fluorescent spot. The solvent front appeared under the ultraviolet light as a bluish-white fluorescent zone with a sharp leading edge. If the pigment zone could not be located under the ultra-

violet lamp, the strip was treated with one of the chromogenic reagents of Wender and Gage[7], exposed to ammonia vapors, or sprayed with Benedict's solution. A bright spot appeared at the pigment zone. The $R_f$ values were calculated as follows:

$$R_f = \frac{\text{distance moved by pigment}}{\text{distance moved by solvent}}$$

These values are tabulated in Table II.

Descending Two-Dimensional Paper Chromatography - Sheets of Whatman No. 1 filter paper 47 x 57 cm. were spotted with solutions of flavonoid compounds in ethanol at a points 8 cm. from each edge. The sheets were suspended in the troughs and allowed to develop as described in the preceding section. The sheets were removed and dried. They were then developed in a different solvent in the direction at right angles to the original development. When this second development was complete, the sheets were removed, dried, and examined.

Hydrolysis of Flavonol Glycosides in Micro-Quantities on Paper - A quantity of solution which contained 0.2 to 0.4 mg. of the glycoside was spotted on the paper strip as described above. The glycoside spot was then held in a stream of the vapor from a boiling solution prepared by adding 15 ml. of concentrated hydrochloric acid to 85 ml. of water. Care was taken to avoid excessive wetting of the paper, since the spot was found to spread when moist. The strip was then chromatographed with one of the following systems: 60% isopropyl alcohol; 40% butanol - 50% water-10% acetic; or 95% ethanol. After drying, the strip was sprayed with Benedict's solution, dried, and heated at 100° in the oven for 5 minutes. The position of the aglycone zone was visible before the heating. After

TABLE II

$R_f$ Values of Flavonoid Compounds

| Compound | Solvent Systems: 1 part chloroform 2 parts Isobutyl Alc. 2 parts water | 22% Isopropyl Alc. 78% Water | Isopro-pyl Alc. 60% | 15% Acetic Acid | 2 parts Heptane 1 part Butanol 4 parts Water (water layer) | 60% Acetic Acid | Colors Produced By Benedict's Solutio: Visible Light | Ultraviolet Light |
|---|---|---|---|---|---|---|---|---|
| **Flavonol Aglycone** | | | | | | | | |
| Gossypetin | - | .06 | .51 | .12 | .07 | .43 | Y | B |
| Kaempferol | .05 | .09 | .77 | .10 | .04 | .50 | Y | OY |
| Morin | .12 | .26 | .58 | .27 | .13 | .68 | Y | OY |
| Nortangeretin | - | .08 | .60 | .10 | .04 | .54 | Y | OY |
| Patuletin | - | .10 | .76 | .10 | .06 | .50 | OY | B |
| Quercetagetin | - | .24 | .61 | .19 | .13 | .63 | Y | OY |
| Quercetin | .05 | .06 | .67 | .07 | .04 | .40 | OY | OY |
| Rhamnetin | .07 | .08 | .73 | .08 | .03 | .60 | OY | OY |
| Robinetin | .05 | .07 | .58 | .08 | .03 | .32 | Y | Y |
| **Flavonol Glycosides** | | | | | | | | |
| Gossypin | - | - | - | - | - | - | -- | Y |
| Gossypitrin | .16 | .13 | .54 | .14 | .17 | .44 | -- | -- |
| Isoquercitrin | .27 | .40 | .79 | .46 | .24 | .74 | OY | OB |
| Quercemeritrin | .28 | .42 | .80 | .45 | .27 | .73 | OY | OB |
| Quercitrin | .54 | .55 | .79 | .46 | .45 | .74 | OY | OB |
| Robinin | .77 | .72 | .76 | .77 | .71 | .84 | Y | Y |
| Rutin | .54 | .60 | .83 | .62 | .45 | .75 | OY | OB |
| Xanthorhamnin | .69 | .66 | .83 | .68 | .58 | .82 | Y | Y |
| **Flavone Aglycones** | | | | | | | | |
| Acecetin | - | .00 | .80 | .00 | .00 | .71 | Y | OB |
| Apigenin | - | .12 | .89 | .15 | .00 | .66 | Y | Y |
| Auranetin | - | - | .99 | .63 | .34 | .90 | -- | W |
| Chrysin | - | .00 | - | .00 | .00 | .75 | Y | B |
| Genkwanin | - | .00 | .78 | .00 | .00 | .72 | Y | Y |
| Isowogonin | .02 | .00 | .87 | .00 | .00 | .81 | YB | B |
| Norwogonin | .11 | .21 | .83 | .26 | .00 | .73 | YB | B |
| Oroxylin A | - | .00 | .88 | .00 | .00 | .84 | Y | B |
| Wogonin | .02 | .00 | .88 | .00 | .00 | .79 | Y | B |

TABLE II (Continued)

| Compound | $R_f$ Values of Flavonoid Compounds | | | | | | Colors Produced By Benedict's Solution* | |
|---|---|---|---|---|---|---|---|---|
| | 1 part Chloroform 2 parts Isobutyl Alc. 2 parts Water | 22% Isopropyl Alc. 78% Water | Isopro-pyl Alc. 60% | 15% Acetic Acid | 2 parts Heptane 1 part Butanol 4 parts Water (Water layer) | 60% Acetic Acid | Visible Light | Ultraviolet Light |
| **Flavanone Aglycones** | | | | | | | | |
| Homoeriodictyol | .32 | .49 | .92 | .55 | .29 | .80 | YB | Y |
| Pentahydroxy flavanone | -- | .57 | .87 | .13 | .00 | .73 | YB | B |
| **Flavanone Glycosides** | | | | | | | | |
| Hesperidin | .80 | .79 | .63 | .82 | .17 | .89 | YB | W |
| Naringin | .81 | .75 | .86 | .80 | .77 | .88 | OY | Y |
| Neohesperidin | .80 | .79 | .88 | .81 | .72 | .90 | Y | Y |
| **Chalcones** | | | | | | | | |
| Hesperidin Methyl chalcone | - | .82 | .95 | .92 | .14 | .89 | Y | W |
| Phloretin | - | .45 | .91 | .42 | .00 | .73 | - | - |
| Trihydroxy chalcone | .08 | .15 | .81 | .19 | .06 | .68 | R | R |
| **Others** | | | | | | | | |
| Esculetin | - | .66 | .73 | .60 | .38 | .74 | Y | Y |
| Pomiferin | - | .00 | .89 | .00 | .00 | .78 | - | B |

* Y - yellow
O - orange
B - brown
W - white
R - red

the heating, the sugar zones were visible as orange spots of cuprous oxide against a blue background.

The aniline hydrogen phthalate reagent of Partridge[81] was also used as a spray for use in detecting the sugar zones. A solution, prepared by dissolving 2 g. of phthalic acid and 1.12 g. of aniline in 25 ml. of water-saturated butanol, was sprayed on the developed strips. When the paper was dried, the sugar zones appeared as faint yellow spots. Examination of the sprayed strip under ultraviolet light revealed brilliant white fluorescent spots at the sugar zones.

## Chromatographic Isolation Procedures

Methylated Cellulose Column - Five grams of "Methocel"* were suspended in 25 ml. of 95% ethanol. The resulting slurry was poured into a chromatographic tube 11 x 130 mm. while a slight suction was applied. When the column of packed material was within 4 cm. of the top of the tube, addition was stopped, and the level of the ethanol was adjusted to within approximately 5 mm. of the top of the packed Methocel. One milliliter of a solution, containing 10 mg. each of rutin and quercetin, was poured on the column. Development with 95% ethanol caused no separation of the bands. Although development of an identical column with benzene caused no separation, the yellow band moved more slowly than with ethanol.

Filter Paper Pulp Column - Schleicher and Schull filter paper pulp was packed into chromatographic columns by two different methods. In the first, the dry pulp was packed into the column with a glass rod used as a tamper. In the second the dry pulp was stirred into water to form a thin slurry which was poured into the tube with slight suction. In both

* Obtained from Dow Chemical Company, Midland, Michigan

methods some heterogeneity of texture in the column resulted. The technique described in the preceding paragraph was used in placing the mixture of rutin and quercetin on the column. Water-saturated butanol was used for development. The flavone band broadened considerably during development and the leading part of the band was a much brighter yellow than the trailing edge. These facts indicated that the quercetin was moving at a faster rate than the rutin. No complete separation of the compounds resulted, however.

Oxidized Cellulose Column - "Polycell-fluf", a product obtained from the oxidation of cellulose with oxides of nitrogen was packed into chromatographic columns in the same manner as described for "Methocel", except that water was used to prepare the slurry. When the solution containing the rutin and quercetin was placed on the column, the color of the pigments became more intense and darker in shade. Development of the column with water-saturated butanol caused the pigment band to spread down the entire length of the column.

Starch Column - A slurry was prepared in the ratio of 1 g. of potato starch, previously ground for several hours in a ball mill, to 1 ml. of butanol saturated with water. This slurry was poured into a chromatographic column and allowed to settle. The excess solvent was removed by gentle suction. After the level of the packed starch had reached an appropriate height, the column was washed with approximately twice its volume of butanol saturated with water, and the pigments placed on the column. The column was developed with water-saturated butanol. No separation of the pigments occurred. Similar results were obtained with rice, tapioca, and corn starch.

## Flavonoid Constituents of Big Bend Locoweed

Two approaches were made toward the identification of the flavonoid constituents of Big Bend Locoweed. In the first, attempts were made to isolate the pigments as pure compounds for characterization. In the second, identification of the flavonoid pigments by chromatographic studies of concentrates was attempted.

Isolation of Flavonoid Pigments - Seventeen kilograms of the ground weed were extracted with 14 l. of boiling water. The extract was separated from the residue by successive filtration through cheese cloth and muslin, and was then clarified in a "Sharples Super-Centrifuge". The extract was concentrated to a thick syrup and extracted with 100 ml. of boiling water in several portions. A small amount of yellow material crystallized from the water solution. This precipitate, removed by filtration and recrystallized once from dilute ethanol, weighed 15 mg.; m. p. 200-5°.

Determination of Properties of Isolated Pigment - Several color tests were applied to the alcoholic solution of the isolated pigment. Reduction with magnesium and hydrochloric acid gave a reddish-orange coloration. With alcoholic ferric chloride, an olive-green color resulted. Both lead acetate and basic lead acetate gave yellow precipitates. Treatment with either ammonium hydroxide or concentrated sulfuric acid resulted in deep yellow solutions.

The ultraviolet absorption spectrum of an ethanol solution was determined with the Beckman Model DU spectrophotometer. A large absorption maximum at 260-265 mμ and on a small one at 350-360 mμ were observed (Figure 2).

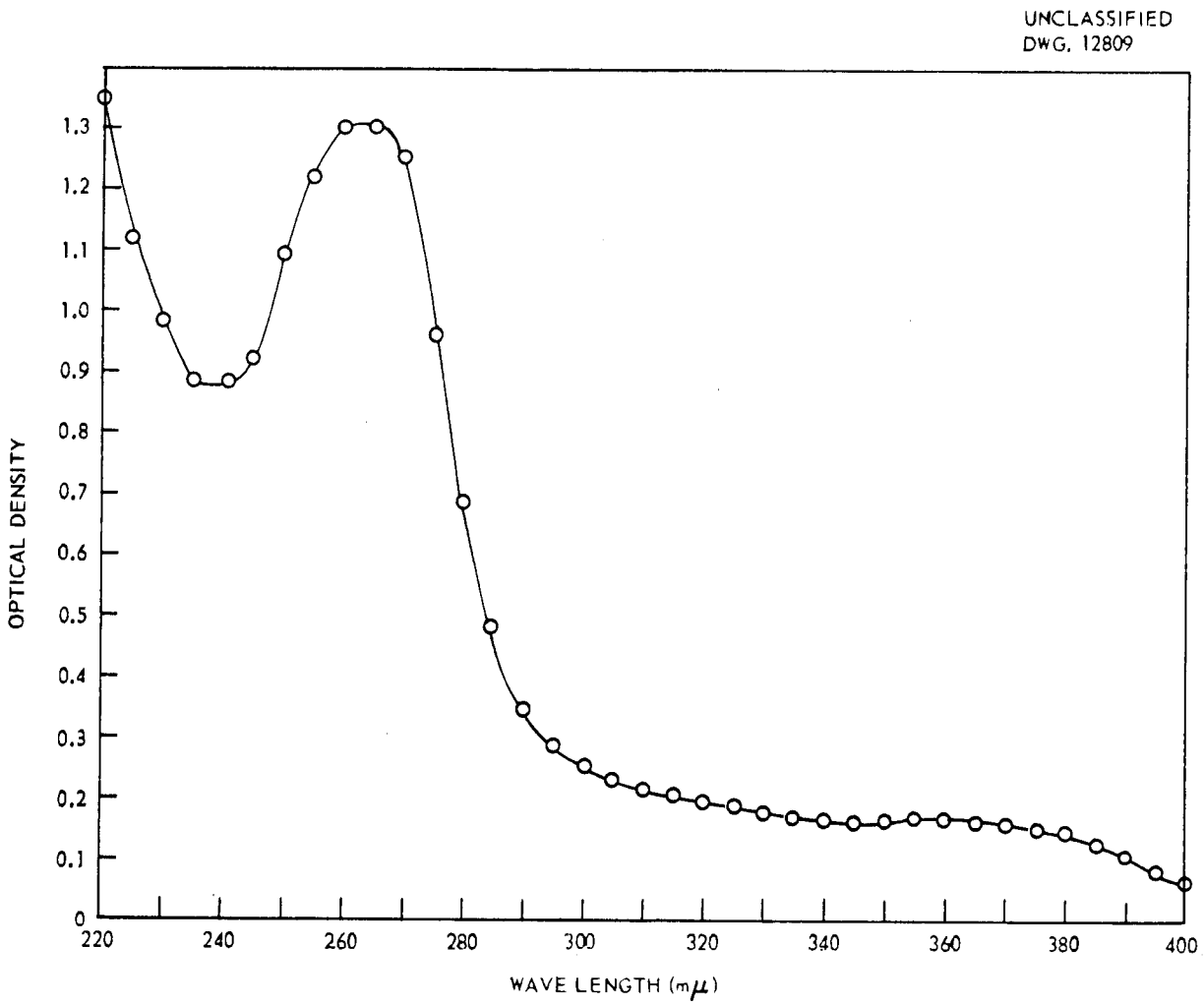



Figure 2

Absorption Spectrum of Substance Isolated from Big Bend Loco Weed

The $R_f$ values of the pigment in three solvent systems as determined by one-dimensional chromatography were: 0.79 in 40% butanol-50% water-10% acetic acid; 0.65 in ethyl acetate, saturated with water; 0.50 in phenol, saturated with water. The pigment exhibited a yellow-brown color when viewed under ultraviolet light after chromatography. When the pigment was treated with the chromogenic spray reagents of Wender and Gage[7] the colors tabulated below were obtained.

| Reagent | Visible Light | Ultraviolet Light |
|---|---|---|
| Basic lead acetate solution | Yellow | Orange |
| Lead acetate solution | Yellow | Orange-Yellow |
| Sodium carbonate solution | Yellow | Yellowish-Brown |
| Alcoholic aluminum chloride | Yellow | Yellow |

Five milligrams of the isolated material were added to 0.5 ml. of 0.6% sulfuric acid and boiled under reflux for 90 minutes. A precipitate separated from the cooled solution. To the supernatant liquid was added an excess of a mixture containing two parts by weight of sodium acetate and three parts of phenylhydrazine hydrochloride. This was allowed to stand in a boiling water bath for 30 minutes. A drop of the liquid was examined under a microscope with the low-power objective. The characteristic crystals of glucosazone were observed.

The amount of precipitate resulting from hydrolysis was too small to make chemical identification possible. A solution of a small amount

of the material in 95% ethanol was subjected to paper chromatography and gave $R_f$ values as follows: 0.79 in ethyl acetate, saturated with water, 0.50 in phenol, saturated with water, and 0.80 in 40% butanol-50% water-10% acetic acid.

Chromatography of Flavonoid Concentrate - Two kilograms of the ground weed were extracted with 4 l. of acetone. The acetone was allowed to evaporate at room temperature, and the residue was taken up in 1 l. of boiling water and filtered while hot. When the solution was concentrated to 70 ml. on a boiling water-bath, a red oil separated, and was removed by filtration. The solution was extracted repeatedly with 20 ml. portions of benzene. The benzene extract gave negative reactions for flavones. The aqueous layer was then exhaustively extracted with 20-ml. portions of ethyl acetate. The ethyl acetate was allowed to evaporate and the resulting syrup was taken up in 3 ml. of 95% ethanol. Ten milliliters of water were added.

Four bands which gave positive flavonoid reactions were observed on paper chromatography in 60% acetic acid. The $R_f$ values of these bands were as follows: 0.41, 0.55, 0.75, 0.86. The colors of these bands in ultraviolet light in the order of increasing $R_f$ values, were as follows: yellow, yellow, brownish-yellow, light yellow. On chromatography in 40% butanol-10% acetic acid-50% water, five such bands were observed. The $R_f$ values of the observed bands were 0.38, 0.50-0.54, 0.79, 0.85, 0.98. Their colors in ultraviolet light, were respectively yellow, brownish-yellow, brownish-yellow, yellow, light yellow.

The solution, concentrated to a volume of approximately 2 ml., was chromatographed on 32 different paper strips with the butanol-acetic acid-

water system. The appropriate bands were cut from each of the strips and the combined zones were extracted with 95% ethanol in a small Soxhlet-type extractor. The pigments have thus been separated for further identification studies.

## The Flavonoid Constituents of Thompson White Seedless Grapes

Concentration of Flavonoid Constituents - A batch of fresh Thompson White Seedless grapes weighing 1127 g. was mashed with a glass rod and liquidized in a Waring Blendor. The homogenate was heated to 80° for two hours and was then stored under toluene for two months. The homogenate was filtered, using Johns-Manville "Celite" as a filter aid.

The residue was extracted with 500 ml. of hot 95% ethanol. This extract was filtered hot. On cooling, a gelatinous precipitate separated and was removed by filtration. The filtrate was allowed to evaporate slowly to a viscous syrup. When this syrup was treated with 300 ml. of water, a grey gelatinous precipitate separated and was removed by decantation of the supernatant liquid. Addition of 600 ml. of cold ethanol to the solution caused precipitation of a red solid which was removed by filtration. After the filtrate was concentrated, an equal volume of water was added to the resulting thick syrup. This solution, immiscible with acetone, was extracted with one 100-ml portion and two 20-ml. portions of acetone. The acetone solution, evaporated to 40 ml. gave strong color reactions for flavonoid compounds.

Paper Chromatography of Flavonoid Constituents - Paper chromatography of the acetone extract with several different solvent systems gave two flavonoid bands. Below is a comparison of the $R_f$ values of these compounds with those of quercetin and rutin. The colors refer to those

observed in ultraviolet light. Other bands which gave no flavonoid reaction were observed on the paper.

| Solvent | Yellow Band | Quercetin | Orange Band | Rutin |
|---|---|---|---|---|
| 40% butanol-10% acetic acid-50% water | 0.77 | 0.78 | 0.55 | 0.55 |
| 60% isopropyl alcohol | 0.69 | 0.67 | 0.82 | 0.83 |
| 15% acetic acid | 0.07 | 0.07 | 0.62 | 0.62 |
| 60% acetic acid | 0.40 | 0.40 | 0.75 | 0.75 |

A potato starch slurry was prepared by adding 50 g. of starch to 150 ml. of n-butyl alcohol saturated with water and allowing it to stand for several hours with occasional shaking. A column, 11 mm. x 130 mm., was packed with this slurry. Ten milliliters of the acetone solution were evaporated to dryness, and the residue was redissolved in 2 ml. of water-saturated butanol. After this solution was placed on the column, water-saturated butanol was used for development. A yellow band moved away from the dark material at the top of the column and was eluted. Paper chromatography of this material showed the presence of three flavonoid materials. Two of these bands appeared from their colors and reactions to be glycosides and the other an aglycone. The $R_f$ values of these bands are tabulated below:

| Solvent | Yellow Band | Brown Band I | Brown Band II |
|---|---|---|---|
| 60% isopropyl alcohol | 0.69 | 0.78 | 0.83 |
| 40% butanol-50% water-10% acetic acid | 0.76 | 0.69 | 0.59 |
| 60% acetic acid | 0.40 | 0.75 | ---- |
| 22% isopropyl alcohol | 0.06 | 0.40 | 0.62 |

Ion Exchange Adsorption of Flavonoid Constituents - A column of "Amberlite" IRC-50(H) was prepared as follows: A 2-in. layer of glass wool was placed in the constricted end of a Pyrex tube (2" x 48"). The glass wool was covered by a 2-in. layer of pure sea sand. A slurry of the resin in distilled water was poured through the tube until the height of the resin column reached 36 in. The column was washed with 2 l. of 10% sulfuric acid, "backwashed" with distilled water and finally "downwashed" with distilled water until the eluate was neutral. A solution, prepared by extraction of 17 kg. of grapes with 8 gal. of boiling water was cooled and passed over the resin at a rate of 8 gal. per hour. After thoroughly washing the column with distilled water, the pigment zone was eluted with 1 l. of ethanol. The column was washed again with water and recharged with 10% sulfuric acid. After the excess acid was washed out with distilled water, the resin was converted to the potassium salt by treatment with a large quantity of a 5% aqueous potassium acetate. The extract, which had been passed through the IRC-50(H) resin, was then passed over the IRC-50(K) resin at the same rate. The column was washed with distilled water. The yellow band at the top of the column was eluted with 1 l. of ethanol. The ethanol eluates from the two treatments were combined.

To the combined eluates were added 10 g. of aluminum chloride hexahydrate. The pH of the solution was adjusted to 6.5 with concentrated ammonium hydroxide. The resulting gelatinous precipitate was removed by filtration and dried at room temperature. This material was stirred into 500 ml. of acetic acid in which had been placed several hundred grams of ice.

After the solution stood for several hours in the cold, a greyish-black precipitate separated and was discarded. After standing in the cold for several days, the solution was diluted with 4 volumes of water and was extracted with 250 ml. of diethyl ether. The water solution, designated as "Fraction WW," gave positive magnesium-hydrochloric acid reduction tests.

The ether extract was washed with dilute sodium bicarbonate and with water to remove acetic acid, and was dried over anhydrous sodium sulfate. It was filtered and concentrated. This material as "Fraction WE," was taken up in 10 ml. of 95% ethanol and set aside for further study.

## Flavonoid Constituents of Tokay Grapes

Concentration of Flavonoid Constituents - A sample of Tokay grapes weighing 2240 g. was homogenized in a Waring Blendor and heated to 80° to stop enzyme activity. The cooled homogenate was stored under toluene in the refrigerator. The solid material was separated by filtration and extracted exhaustively with hot acetone. The acetone extract was filtered and was concentrated. The resulting thick syrup was extracted with 750 ml. of boiling water. The cooled solution was extracted ten times with ethyl acetate in 40-ml. portions. The ethyl acetate extract

was concentrated in a current of warm air and the residue was taken up in 50 ml. of 95% ethanol. One hundred milliliters of water was added to the solution. The solution was extracted with high-boiling petroleum ether until the hydrocarbon layer was colorless. The residue from the concentrated ether solution was dissolved in 15 ml. of 95% ethanol and diluted with 15 ml. of water. When this solution was used for paper chromatography, three pigment bands were observed. One was yellow under ultraviolet light, and two were brownish-yellow. The $R_f$ values in various solvents are shown below.

| Solvent | Yellow Band | Brown Band I | Brown Band II |
|---|---|---|---|
| 60% acetic acid | 0.41 | 0.73 | 0.83 |
| 15% acetic acid | 0.07 | 0.45 | 0.51 |
| 50% butanol-10% acetic acid-50% water | 0.77 | 0.70 | 0.75 |
| 60% isopropyl alcohol | 0.65 | 0.80 | None |

Concentration of Flavonoid Constituents Using Ion-Exchange Resins - The ion-exchange isolation technique was applied to the concentration of the flavonoid fraction of Tokay grapes. Sixteen liters of a filtered, aqueous extract of 21 kg. of Tokay grapes were passed over the IRC-50(H) resin which was prepared as described on page 42. The adsorbed material was eluted with ethanol. The aqueous effluent from the hydrogen resin was passed over the potassium form of the resin as described on page 42. The ethanol eluate was combined with that from the hydrogen form of the resin.

To the combined eluates were added 10 g. of aluminum chloride hexahydrate. After the pH was adjusted to 7 with concentrated ammonium hydroxide, the gelatinous precipitate was filtered off and dried in the air at room temperature. The precipitate was dissolved in excess concentrated hydrochloric acid containing an equal weight of ice.

The cold hydrochloric acid solution was extracted with ethyl acetate until the organic layer was colorless. The ethyl acetate solution was concentrated *in vacuo* to 10 ml. and diluted with 50 ml. of water. After this solution had been extracted exhaustively with ether, it gave a positive magnesium-hydrochloric acid reduction test. This fraction was designated "RW." The ether solution, designated "RE", also showed positive reactions for flavonoid pigments. The aqueous fraction "RW" was allowed to evaporate slowly in a beaker. After evaporation of the solvent, there remained in the center of the bottom of the beaker a black deposit of a gum, and surrounding this, a yellow crystalline solid. The yellow solid, which was removed by carefully scraping it away from the gum, weighed about 50 mg. Paper chromatography yielded the following $R_f$ values: 0.75 in 40% butanol-10% acetic acid-50% water, 0.61 in phenol saturated with water, 0.83 in 60% acetic acid, and 0.51 in 15% acetic acid.

The ether solution, "RE", was allowed to evaporate to approximately 1 ml., when sufficient water was added to cause cloudiness. A yellowish-grey precipitate separated after vigorous shaking. This precipitate was separated by centrifugation and extracted with 2 ml. of ethanol. The residue gave no positive tests for flavonoid material. The ethanol solution was recombined with the supernatant solution from the precipitate which gave positive tests for flavonoid material. The ethanol solution

was recombined with the supernatant solution from the precipitate which gave positive flavonoid tests. Paper chromatography showed at least two flavonoid constituents to be present. In two of the solvent systems, three bands appeared. The $R_f$ values are tabulated below:

| Solvent | $R_f$ Values |
|---|---|
| 40% butanol-10% acetic acid-50% water | 0.46, 0.69, 0.85 |
| 60% acetic acid | 0.74, 0.81 |
| Phenol saturated with water | 0.36, 0.65, 0.82 |
| 15% acetic acid | 0.53, 0.60 |

To 1 ml. of the "RE" fraction was added 0.25 ml. of concentrated hydrochloric acid. After this solution was boiled for 90 minutes and chilled, a reddish-brown precipitate formed. The supernatant liquid was subjected to paper chromatography. The $R_f$ values appear below:

| Solvent | $R_f$ Values |
|---|---|
| 60% acetic acid | 0.48 |
| Phenol saturated with water | 0.39 |
| 15% acetic acid | 0.09 |

## 2,4-Dinitrophenylhydrazones of Flavanones

A stock solution of 2,4-dinitrophenylhydrazine reagent was prepared by adding to a mixture of 1 g. of 2,4-dinitrophenylhydrazine and 2 ml. of concentrated sulfuric acid sufficient 95% ethanol to dissolve the solid.

Twenty milligrams of the appropriate flavanone were dissolved in 2 ml. of the stock reagent solution. After the solution was heated to boiling in a water bath, it was allowed to cool slowly. The dinitrophenylhydrazone which separated on standing was removed by filtration through sintered glass, and recrystallized from 1,4-dioxane and water. The solid, washed with cold ethanol and ether, was dried at 100° for two hours.

The derivatives, which were sparingly soluble in ethanol, ether, and acetone were very soluble in 1,4-dioxane. Although the compounds were red in the solid state, solutions of them in organic solvents were yellow. These compounds act as acid-base indicators, exhibiting a yellow color in acid and a dark violet in base.

The absorption spectra of 2,4-dinitrophenylhydrazones of liquiritigenin, hesperitin, and 7-hydroxy flavanone in 95% ethanol were determined with a Beckman Model DU spectrophotometer. The spectra for solutions containing 1 mg. per 100 ml. are shown in Figures 3, 4, and 5. Absorption bands at about 400 mμ were observed with each of the compounds.

The 2,4-dinitrophenylhydrazones were subjected to one-dimensional paper chromatogtaphy by the method described previously. The solvent system used contained 50% water, 30% 1,4-dioxane, and 20% glacial acetic acid by volume. Table III shows the $R_f$ values together with the melting

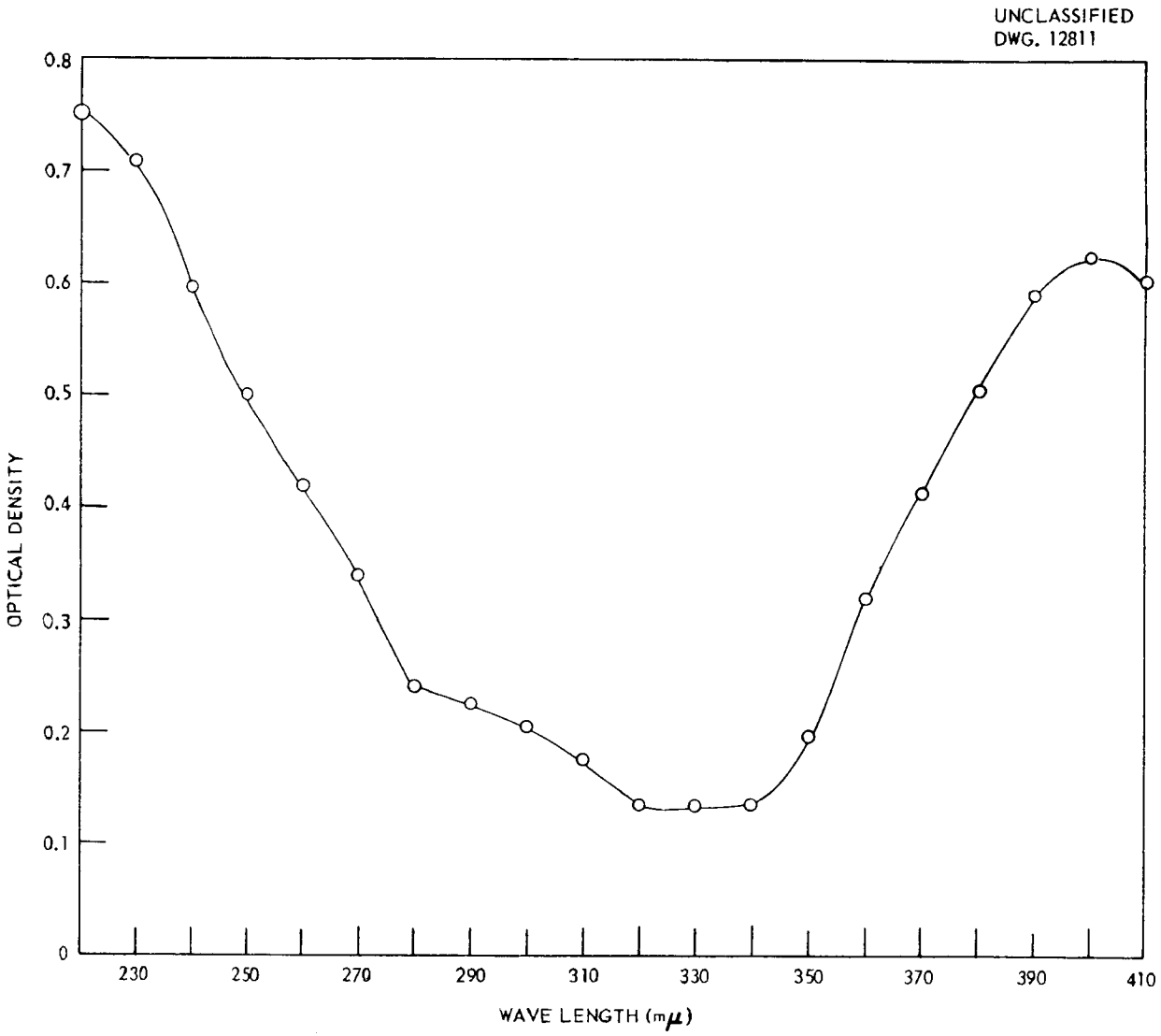



Figure 3
Absorption Spectrum of Liquiritigenin-
2,4-dinitrophenylhydrazone

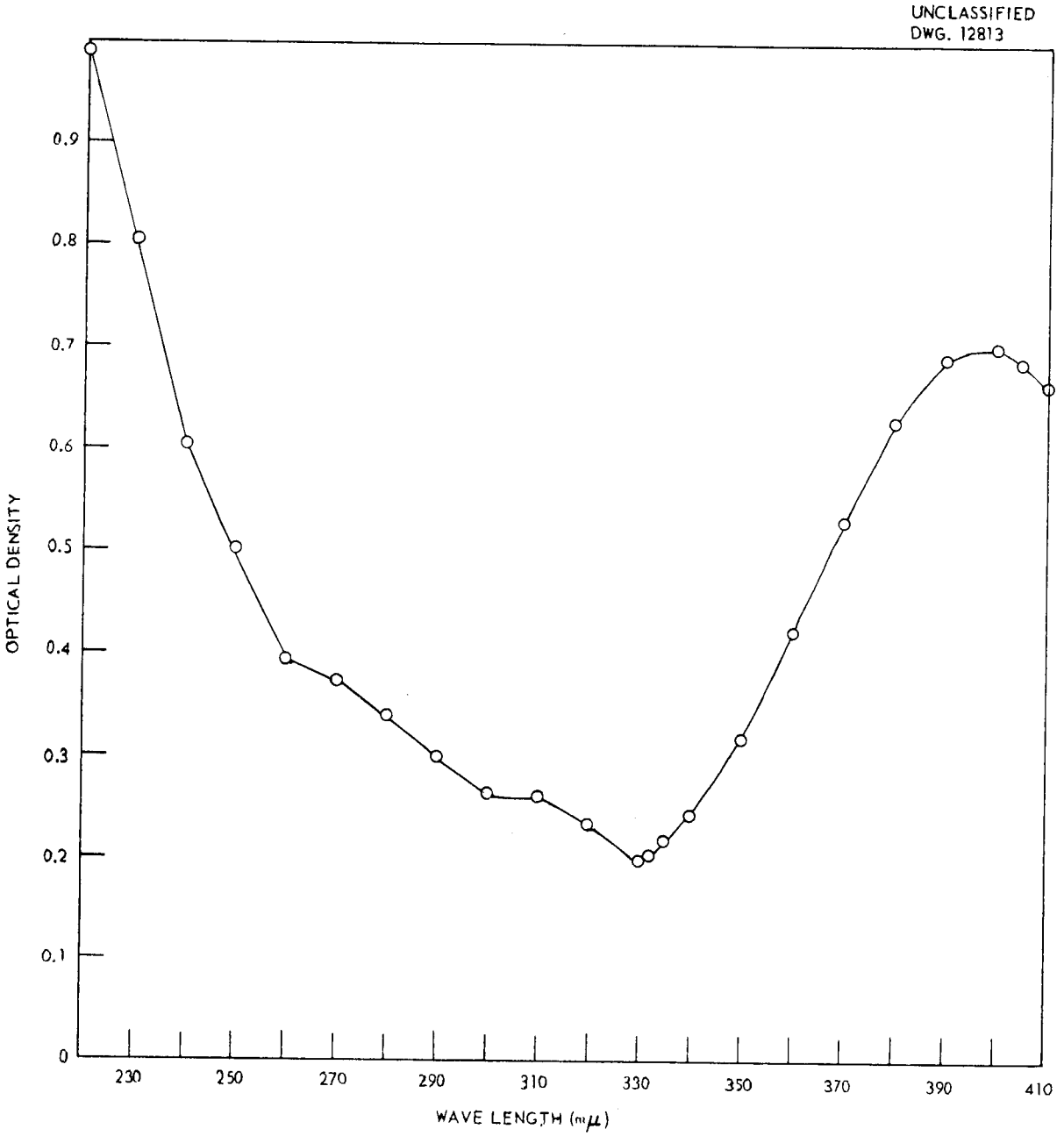



Figure 4

Absorption Spectrum of Hesperitin-2,4-dinitrophenylhydrazone

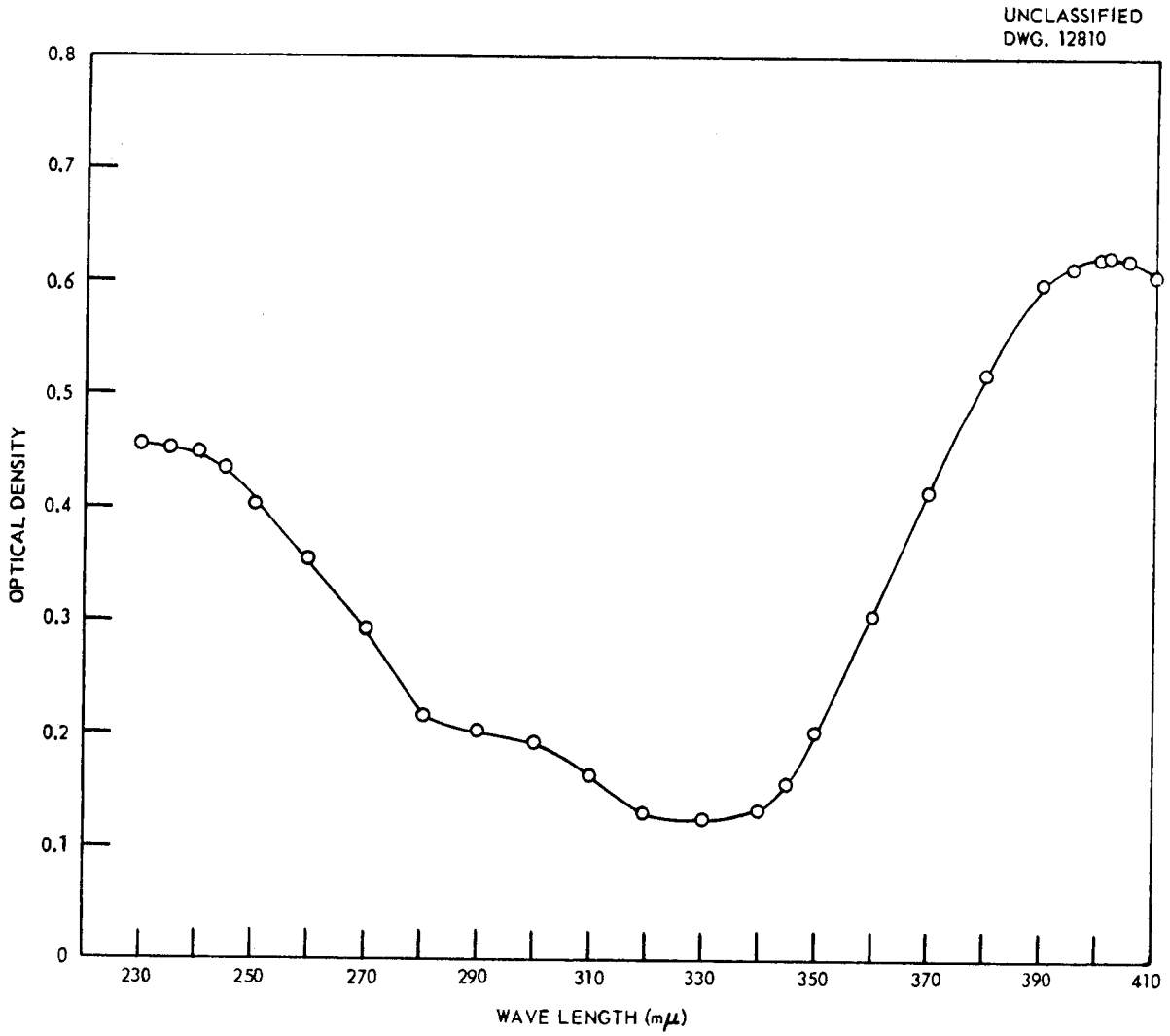



Figure 5

Absorption Spectrum of 7-Hydroxyflavanone-2,4-dinitrophenylhydrazone

points and nitrogen analyses. The melting points were determined with a Fisher-Johns melting point aparatus. Nitrogen was determined by the Dumas method using 10-mg. samples.

Table III

Properties of the 2,4-Dinitrophenylhydrazones of Some Flavanones

| 2,4-Dinitrophenyl-hydrazone of: | M.P. | $R_f$ | % Nitrogen Calcd. | % Nitrogen Found |
|---|---|---|---|---|
| Butin | 249-7° | 0.80 | 12.39 | 12.29 |
| 4',7-dihydroxy-3',5'-dimethoxyflavanone | 250° | 0.87 | 11.32 | 10.41 |
| 4',7-dihydroxy-3'methoxy-flavanone | 255° (d) | 0 | 12.01 | 12.36 |
| Hesperitin | 293° (d) | 0.81 | 11.61 | 11.13 |
| Homoeriodictyol | 295° (d) | 0 | 11.61 | 11.29 |
| 7-Hydroxyflavanone | 272° | 0 | 13.33 | 12.16 |
| Liquiritigenin | 258-9° | 0 | 12.83 | 12.90 |
| Naringin | 246-7° | 0.98 | 7.37 | 6.82 |

## Isotopic Exchange Reactions

### Friedel-Crafts Acylation Reaction

Radioactivity Analyses - The precise assays for carbon-14 reported in this thesis were performed by a procedure described by Neville[82]. In this method, 2- to 20-mg. samples of the radioactive compounds were oxidized by the Van Slyke-Folch[83] procedure to carbon dioxide which was collected in an ion chamber[84]. The ion current produced by the radioactivity was measured with a vibrating- reed electrometer (Model 30, Applied Physics Corp., Pasadena, California), and recorded on a Brown recorder. The ion current in amperes, was converted to millicuries by use of the factor $2.0 \times 10^8$ millicuries/ampere.

For rapid evaluation of the relative radioactivities of the various organic compounds prepared, a thin sample of the material was distributed over a known area of a sample card. The approximate radioactivity level was then determined with a thin window Geiger-Muller tube and scaler.

In the experiments reported below the radioactivities will be reported as millicuries per mole (mc/mole) since this form is usually of greater significance than is radioactivity per unit weight (specific activity).

Preparation of Acetyl- 1-$C^{14}$ Chloride - A 50-g. portion of acetic-1-$C^{14}$ acid* was treated with 25 ml. of phosphorous trichloride. After the reaction mixture stood overnight and was heated under reflux for two hours, the acetyl chloride was distilled. The crude product was treated with several drops of dimethylaniline and redistilled. The yield of acetyl-1-$C^{14}$ chloride was

* Kindly supplied by Mr. D. N. Hess, Chemistry Division, Oak Ridge National Laboratory.

37 g. or 57% of the theoretical amount; m. p. 52°. Radioactivity assay: 2 mc./mole.

Preparation of Benzoyl-7-$C^{14}$ - A 50-g. portion of benzoic-7-$C^{14}$ acid* whose radioactivity was 6.29 mc./mole, was treated with 22 ml. of thionyl chloride. The reaction mixture, which was boiled for one hour and allowed to stand overnight, was heated to remove the excess thionyl chloride. The benzoyl chloride was distilled at 194-8°. The weight of product was 42 g. or 73% of the theoretical amount.

Preparation of 2,4,6-Trihydroxyacetophenone - Five grams of phloroglucinol were added to 3 g. of acetonitrile and 2 g. of anhydrous zinc chloride in 50 ml. of ether. Dry hydrogen chloride was passed through the solution for 45 minutes. After standing for several hours, the reaction mixture was treated with 25 ml. of water and extracted with ether. The water layer was boiled for 30 minutes and cooled. The 2,4,6-trihydroxyacetophenone which crystallized was filtered off, dissolved in hot water, and treated with decolorizing charcoal. The crystalline ketone removed by filtration from the cooled solution weighed 3.2 g., or 80% of the theoretical amount; m. p. 216-8° [85].

Preparation of 2,4,6-Trinitrobenzophenone- To 5 g. of 2,4,6-trinitrobenzoyl chloride in 25 ml. of benzene were added slowly 10 g. of aluminum chloride. A vigorous evolution of hydrogen chloride occurred. The reaction mixture was stirred for 30 minutes at room temperature and was poured into a dilute hydro-

* Kindly supplied by Mr. W. J. Skraba, Chemistry Division, Oak Ridge National Laboratory.

chloric acid-ice mixture. The solid was filtered off and crystallized twice from ethanol. The weight of the 2,4,6-trinitrobenzophenone was 2.5 g. or 43% of the theoretical amount; m. p. 194°.

| | | C | H |
|---|---|---|---|
| Anal.* | Calcd. for $C_{13}H_7O_7N_3$ | 49.20% | 2.21% |
| | Found | 50.17% | 2.44% |

Reaction Conditions of Attempted Exchanges - The conditions under which the attempted exchange reactions were carried out are summarized in Table IV. A typical reaction may be illustrated by the attempted exchange between acetophenone and acetyl chloride. Into a four-necked flask, equipped with stirrer, thermometer, dropping funnel, and water condenser with drying tube, were placed 11.4 g. of anhydrous aluminum chloride. Fifteen milliliters of dry nitrobenzene containing 5 ml. of acetophenone were added slowly with cooling. To this cool solution, 3.2 ml. of radioactive acetyl chloride were added. The reaction mixture was allowed to stand at room temperature with stirring for 70 hours. Dilute hydrochloric acid, containing ice, was poured into the reaction mixture. The resulting solution was extracted with ether and the ether was boiled off on the steam-bath. A 3-ml. portion of a 2,4-dinitrophenylhydrazine solution was added to the residual liquid. The derivative, which was washed several times with cold ether and dried, weighed 0.1 g.; m. p. 247-9°.[86] Examination for radioactivity with a Geiger-Muller counter revealed no exchange had occurred.

* All carbon, hydrogen, and nitrogen analyses reported in this thesis were done by the Galbraith Microanalytical Laboratories, Knoxville, Tennessee.

TABLE IV

Results of Friedel-Crafts Acylation Studies

| Expt. No. | Ketone | Molar Proportion | Labeled Acyl Compound | Molar Proportion | Catalyst | Molar Proportion | Solvent | Conditions | Activity * in Ketone | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | $C_6H_5COCH_3$ | 1 | $CH_3COCl$ | 1 | $AlCl_3$ | 2 | $C_6H_5NO_2$ | 70 hours at 25° followed by 23 hrs at 112°. | - | |
| 2 | $C_6H_5COCH_3$ | 1 | $CH_3COCl$ | 1 | $AlCl_3$ | 2 | $CS_2$ | 92 hours at 25°. | - | |
| 3 | $C_6H_5COCH_3$ | 1 | $CH_3COCl$ | 1 | Gaseous HCl | | None | Sealed tube at 110° for 30 hours. | ? | No ketone recovered. |
| 4 | $C_6H_5COCH_3$ | 1 | $CH_3COCl$ | 1 | $AlBr_3$ | 2 | $CS_2$ | 25 hrs. at 25°, anhydrous 16 hrs. at 25°, trace of $H_2O$. | - | |
| 5 | $C_6H_5COCH_3$ | 1 | $CH_3COCl$ | 1 | $AlCl_3$ | 4 | $CS_2$ | 20 hours 25°. | - | |
| 6 | p-$CH_3OC_6H_4COCH_3$ | 1 | $CH_3COCl$ | 1½ | 85% $H_3PO_4$ | trace | None | 3 hours at reflux. | - | |
| 7 | p-$CH_3OC_6H_4COCH_3$ | 1 | $CH_3COCl$ | 1 | 85% $H_3PO_4$ | " | " | 16 hours at 25° followed by 3 hours at reflux. | - | |
| 8 | 2,4-$(OH)_2C_6H_3COCH_3$ | 1 | $CH_3COCl$ | 1 | Conc. $H_2SO_4$ | " | " | Warmed to 50°. Allowed to cool to 25°. | - | |
| 9 | 2,4-$(OH)_2C_6H_3COCH_3$ | 1 | $CH_3COCl$ | 1 | $AlCl_3$ | 3 | $CS_2$ | 40° for 1 hr followed by 5 days at 25°. | + | See page for explanation. |
| 10 | 2,4-$(OH)_2C_6H_3COCH_3$ | 1 | $CH_3COCl$ | 1 | $SnCl_4$ | 5 | $CS_2$ | 24 hours at 25°. | ? | No pure ketone isolated. |
| 11 | $C_6H_5COCH_3$ | 1 | $CH_3COCl$ | 2 | $AlCl_3$ | 3 | $C_6H_5NO_2$ | 3 months at 25°. | - | |
| 12 | $(C_6H_5)_3COC_6H_5$ | 1 | $C_6H_5COCl$ | 1 | $AlCl_3$ | 2 | $C_6H_5NO_2$ | 1 hr at 120° followed by 3 days at 25°. | ? | No ketone could be isolated. |

TABLE IV (Continued)

Results of Friedel-Crafts Acylation Studies

| Expt. No. | Ketone | Molar Proportion | Labeled Acyl Compound | Molar Proportion | Catalyst | Molar Proportion | Solvent | Conditions | Activity* in Ketone | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|
| 13 | $C_6H_5COC_6H_5$ | 1 | $C_6H_5COCl$ | 1 | $AlCl_3$ | 3 | $C_6H_5NO_2$ | 24 hours at 25°. | - | |
| 14 | $C_6H_5COC_6H_5$ | 1 | $C_6H_5COCl$ | 1 | $AlCl_3$ | 3 | $CS_2$ | 24 hours at 25°. | - | |
| 15 | $C_6H_5COC_6H_5$ | 1 | $C_6H_5COCl$ | 1 | 85% $H_3PO_4$ | trace | none | 45 minutes at 100° followed by 12 hrs at 25°. | - | |
| 16 | 2,4,6-$(OH)_3$ $C_6H_2COCH_3$ | 1 | $CH_3COCl$ | 1 | $AlCl_3$ | 3 | $CS_2$ | 24 hours at 25°. | - | |
| 17 | p-$CH_3OC_6H_4COCH_3$ | 1 | $CH_3COCl$ | 1 | $AlCl_3$ | 3 | $CS_2$ | 48 hours at 25°. | - | |
| 18 | o-$HOC_6H_4COCH_3$ | 1 | $CH_3COCl$ | 1 | $AlCl_3$ | 3 | $CS_2$ | 11 days at 25°. | - | |
| 19 | 2,4,6-$(OH)_3$ $C_6H_2COCH_3$ | 1 | $CH_3COCl$ | 1 | $AlCl_3$ | 3 | $Cl_2HC-CHCl_2$ | 7 days at 25°. | - | |
| 20 | 2,4-$(OH)_2C_6H_2$ $COCH_3$ | 1 | $CH_3COOH$ | 15 | 48% HBr | 11 | None | 12 hours at 25°. | - | |
| 21 | $C_6H_5COC_6H_5$ | 1 | $C_6H_5COCl$ | 1 | Conc. $H_2SO_4$ | $\frac{1}{2}$ | $Cl_2HC-CHCl_2$ | 3 hours at 120°. | - | |
| 22 | 2,4,6-$(NO_2)_3$ $C_6H_3COC_6H_5$ | 1 | $C_6H_5COCl$ | 1 | $AlCl_3$ | 3 | $Cl_2HC-CHCl_2$ | 24 hours at 25°. | - | |

*This minus sign indicates that no activity was found in the product.

Attempted Identification of Compound Isolated from Experiment 9-

A product which contained radioactivity was isolated from the reaction mixture in the attempted exchange of 2,4-dihydroxyacetophenone, (m. p. 144°) with acetyl chloride. Crystallization of this product from aqueous ethanol yielded white needles, m. p. 138°, soluble in ethanol, ether, and benzene. The mixed melting point with authentic 2,4-dihydroxyacetophenone was 90-110°. The 2,4-dinitrophenylhydrazone of each compound melted at 242-5°.

| | | C | H | N |
|---|---|---|---|---|
| Anal: | Calcd. for $C_8H_8O_3$(2,4-dihydroxyacetophenone): | 63.15% | 5.26% | - |
| | Found: (1) | 62.88% | 5.56% | - |
| | (2) | 60.85% | 5.38% | - |
| | (3) | 61.58% | 5.32% | |

| | |
|---|---|
| Calcd. for $C_{14}H_{13}O_4N_3$(p-nitrophenylhydrazone of 2-4-dihydroxyacetophenone) | 14.62% |
| Found | 12.77% |

Paper chromatography in water-saturated butanol of the isolated material, compared with authentic 2,4-dihydroxyacetophenone, yielded $R_f$ values of 0.98 for both compounds. Its $R_f$ value in 50% acetic acid was 0.88 as compared to 0.94 for authentic 2,4-dihydroxyacetophenone. Reaction of the zones with alcoholic ferric chloride solution yielded a red-brown spot for the unknown material and a violet spot for 2,4-dihydroxyacetophenone.

The molecular weight as determined by the Rast camphor method was 142. The ebullioscopic determination, using benzene as the solvent, yielded a molecular weight value of 143. The molecular weight of 2,4-dihydroxyacetophenone is 152.

The ultra-violet absorption spectra of the two compounds are shown in Figure 6. The spectra were determined with a Beckman Model DU spectrophotometer. The infrared spectrum* determined with a Perkin-Elmer Model 12C instrument, showed bands at 12.75, 11.75, 10.50 and 9.5 microns which were not observed for 2,4-dihydroxyacetopheone.

The compound had a radioactivity of 2.6 mc./mole based on a molecular weight of 143.

A portion of the radioactive compound weighing 100 mg. was added to a solution of 20 ml. of ethanol, 2 ml. of concentrated hydrochloric acid and 2 ml. of water. After the mixture was allowed to stand overnight, it was heated under reflux for one hour and the solvent was evaporated on the steam bath. The residue was taken up in fresh ethanol. This solution was filtered through a pad of decolorizing charcoal and the ethanol was evaporated. The white, crystalline material weighed 50 mg.; m. p. 142-3°; mixed m. p. with authentic 2,4-dihydroxyacetophenone 143-4°; mixed m. p. with original radioactive compound 90-100°. This material, identified as 2,4-dihydroxyacetophenone, was non-radioactive.

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*The author wishes to express his gratitude to Dr. Fred Vaslow, Biology Division, Oak Ridge National Laboratory for his kind assistance in the determination of the spectrum.

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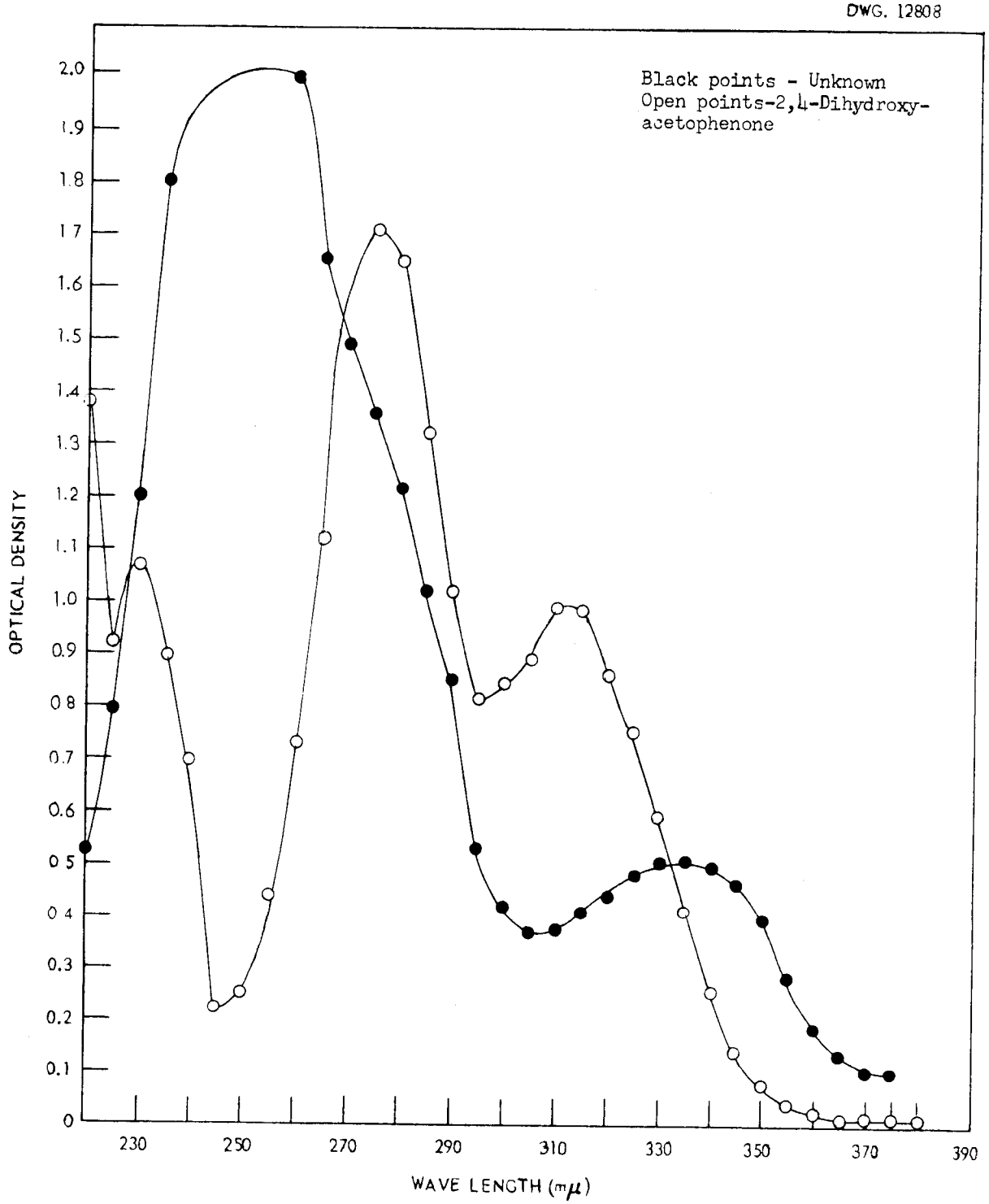



Figure 6

Absorption Spectrum of 2,4-Dihydroxyacetophenone and Substance from Expt. 9

## Carbonyl-Methylene Condensation Reactions

Preparation of Benzaldehyde-7-$C^{14}$ - A sample of benzoic acid weighing 55.5 mg. and of an activity of approximately 2.5 c./mole was dissolved in 7 ml. of ethyl benzoate. This solution, dissolved in ether, was added dropwise to an ether solution containing excess lithium aluminum hydride. After 30 minutes, 22 ml. of non-radioactive benzyl alcohol were added to the mixture. The excess lithium aluminum hydride was decomposed with water and the mixture was acidified with hydrochloric acid. The ether layer was separated and dried over anhydrous sodium sulfate. After the ether was evaporated, the benzyl alcohol was distilled through a 5-ft. Podbielniak column; b. p. 70°/2mm. Two 15-ml. portions of non-radioactive benzyl alcohol were subsequently distilled through the column. The combined benzyl alcohol distillates, weighing 55 g. were assayed for radioactivity. The radioactivity of 1.5 mc/mole demonstrated a radiochemical yield of 67%.

A solution of 14.3 g. of chromium trioxide in 71 ml. of water was added slowly, with stirring to 20 ml. of benzyl alcohol suspended in 107 ml. of water. The mixture, cooled to 20°, was treated with a solution of 12.9 ml. of concentrated sulfuric acid in 71 ml. of water. The solution was stirred vigorously at room temperature for 1 hour and extracted with ether. The ether layer was washed with water, saturated sodium bicarbonate solution, and finally water. The dried ether solution was distilled through a 6-in. Vigreaux column. The fraction boiling

at 28°/1 mm. was collected. The yield was 15 g. or 77% of the theoretical amount. The 2,4-dinitrophenylhydrazone melted at 237° [87]. Radioactivity assay: 1.49 mc/mole.

In a second similar preparation from benzyl alcohol of higher molar radioactivity, the product was extracted into chloroform and shaken with saturated sodium bisulfite solution. The weight of the benzaldehyde-sodium bisulfite addition product corresponded to 86% of the theoretical yield. Radioactivity assay: 2.32 mc/mole. Radioactivity assay of benzaldehyde 2,4-dinitrophenylhydrazone: 2.34 mc/mole.

Both the pure benzaldehyde and the benzaldehyde-sodium bisulfite addition product were used in the exchange reaction described below.

Preparation of 2,4-Dinitrostilbene - A 25-g. portion of 2,4-dinitrotoluene and 16 ml. of benzaldehyde were heated to 140° and treated with 28 drops of piperidine. The mixture was heated at 140° for 2 hours. The resulting solid product was washed with cold benzene and methanol and crystallized from glacial acetic acid. The yield of 2,4-dinitrostilbene was 24.8 g. or 67% of the theoretical amount; m. p. 143° .

Preparation of 8-Phenylbenzofulvene - A mixture of 1.3 g. of indene, 13 g. of benzaldehyde, 2 g. of potassium hydroxide, and 50 ml. of methanol was heated under reflux for 4 hours. When water was added, a dark oil separated which was extracted into toluene and crystallized successively from xylene and methanol. The yield was 4 g. or 17.5% of the theoretical amount; m. p. 130° [88]. A second crop of crystals, less pure than the first, was recovered from the mother liquor.

The preparation of the dibromide of this compound was attempted. A small sample was dissolved in carbon tetrachloride and treated with a solution of bromine in carbon tetrachloride. As the bromine solution was added, hydrogen bromide was evolved. A yellow oil which could not be crystallized remained from evaporation of the solvent.

Preparation of 2'-Hydroxybenzalacetophenone- A mixture of 10 g. of o-hydroxyacetophenone, 7.8 g. of benzaldehyde, 4.5 g. of sodium hydroxide, 20 ml. of water, and 40 ml. of ethanol was stirred for five hours at room temperature. The mixture was acidified and water was added. The yellow material was filtered off, crystallized twice from 95% methanol and dried. The yield of 2'-hydroxybenzalacetophenone was 3.06 g. or 18.6% of the theoretical amount; m. p. 86-7° [89].

Exchange Reactions with the $\alpha,\beta$-Unsaturated Carbonyl Compounds - All of the attempted exchange reactions in this series were carried out similarly. The results and the conditions under which these reactions were carried out are tabulated in Table V. Two examples will be given here in detail.

To a 0.98-g. sample of benzalacetophenone, dissolved in 30 ml. of ethanol were added 2 ml. of 10% sodium hydroxide solution and 0.5 ml. of carbonyl-labeled benzaldehyde. The solution was stirred for 60 hours at room temperature. Water was added and the benzalacetophenone was allowed to crystallize. The solid was removed by filtration and crystallized several times from ethanol. It was dried overnight at 2 mm. pressure in an Abderhalden drying pistol at room temperature.

TABLE V

Conditions and Results of Exchange Reactions on $\alpha,\beta$-Unsaturated Compounds

| Exp. No. | $\alpha,\beta$-Unsaturated Ketone | Grams Ketone | Grams Benzaldehyde or Bisulfite Addn. Comp.* | Catalyst | Amount of Catalyst | Ml. of Water | Ml. of Alcohol | Activity of Benzaldehyde (mc/mole) | Activity of Isolated Ketone | Hours of Reaction |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Benzalacetophenone | 1 | 0.5 | NaOH | 0.1 g. | 2 | 30 | 1.489 | 0.441 | 24 |
| 2 | Benzalacetophenone | 0.98 | 0.5 | NaOH | 0.2 g. | 2 | 30 | 1.489 | 0.701 | 60 |
| 3 | Dibenzalacetone | 0.5 | 0.5 | NaOH | 0.1 g. | 2 | 30 | 1.489 | 0.135 | 24 |
| 4 | Dibenzalacetone | 0.5 | 0.45* | NaOH | 0.3 g. | 2 | 30 | 2.320 | 0.457 | 24 |
| 5 | 2'-Hydroxybenzalacetophenone | 1.1 | 0.5 | NaOH | 0.3 g. | 2 | 30 | 1.489 | 0.051 | 24 |
| 6 | 2'-Hydroxybenzalacetophenone | 0.5 | 0.5* | NaOH | 0.2 g. | 2 | 30 | 2.320 | 0.137 | 24 |
| 7 | 2'-Hydroxybenzalacetophenone | 1.1 | 0.5 | NaOH | 0.4 g. | 2 | 30 | 1.489 | 0.130 | 60 |
| 8 | Benzalacetophenone | 0.5 | 0.25 | None | - | 0 | 0 | 2.320 | 0 | 72 |
| 9 | Benzalacetophenone | 0.5 | 0.25 | Piperidine | 1 drop | 0 | 0 | 1.489 | 0 | 24 |
| 10 | Cinnamalacetophenone | 0.56 | 0.5* | NaOH | 0.2 g. | 2 | 30 | 2.320 | 0 | 24 |

* Denotes benzaldehyde addition compound.

The recovery of pure benzalacetophenone was 0.35 g. or 70% of the theoretical amount; m. p. 58°; mixed m. p. with the starting material, 58°. Radioactivity assay: 0.701 mc/mole.

A 0.5-g. sample of dibenzalacetone was added to 20 ml. of methanol, 2 ml. of water, and 0.3 g. of sodium hydroxide. When the sodium hydroxide had dissolved and the solution had cooled, 0.45 g. of labeled benzaldehyde-sodium bisulfite compound was added. The solution was stirred at room temperature for 24 hours. Water was added and the ketone was allowed to crystallize. The solid was removed, crystallized several times from methanol, and dried. The yield was 0.33 g. or 66% of the theoretical amount; m. p. 111° [90]. Radioactivity assay: 0.457 mc/mole.

Exchange of Benzaldehyde with 2,4-Dinitrostilbene- A sample of 2,4-dinitrostilbene weighing 0.63 g. and 0.25 ml of radioactive benzaldehyde were mixed in a flask. One drop of freshly distilled, dried piperidine and one drop of water were added. The mixture was heated in an oil-bath at 140° for 2 hours under a reflux condenser. When the liquid was cooled, a dark solid which had formed was removed and washed 3 times with 10-ml. portions of cold methanol. The resulting yellow solid was crystallized from benzene and dried. The recovery was 0.33 g. or 52% of the theoretical amount; m. p. 143°; mixed m. p. with initial material 142-3°. Radioactivity assay: 0.032 mc/mole.

The results and reaction conditions of several experiments with 2,4-dinitrostilbene are compiled in Table VI.

TABLE VI

Conditions and Results of Exchange Reactions with 2,4-Dinitrostilbene

| Exp. No. | Grams of Stilbene | Grams of Benzaldehyde | Catalyst | Amount of Catalyst | Hours of Reaction | Bath Temp. | Activity of Benzaldehyde (mc./mole) | Activity of Stilbene (mc./mole) | Remarks |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 2 | Piperidine | 5 drops | 2 | 140° | 2.320 | 1.047 | Traces of acetic acid and water present. |
| 2 | 1.27 | 0.5 | Piperidine | 1 drop | 2 | 140° | 1.489 | 0 | Water was excluded from the reaction. |
| 3 | 0.63 | 0.25 | Piperidine | 4 ml. | 4 | 110° | 1.489 | No stilbene could be isolated | " |
| 4 | 0.63 | 0.25 | Piperidine | 4 ml. | 20 | 25° | 1.489 | 0 | " |
| 5 | 0.63 | 0.25 | Piperidine Acetate | 20 mg. | 2 | 140° | 1.489 | 0 | " |
| 6 | 0.63 | 0.25 | Piperidine | 1 drop | 2 | 140° | 1.489 | 0.032 | 1 drop of water added. |

Exchange of Benzaldehyde with 8-Phenylbenzofulvene - One gram of 8-phenylbenzofulvene and 0.2 g. of potassium hydroxide were dissolved in 20 ml. of methanol. After the potassium hydroxide had dissolved, 1 ml. of benzaldehyde (2.33 mc./mole) was added. The mixture was heated under reflux for 4 hours. One volume of water was added and the mixture was acidified with hydrochloric acid. A brown oil which separated was removed, dissolved in acetone, and allowed to crystallize. The yellow solid resulting was dissolved in benzene. When the benzene solution was chromatographed over a column of alumina, a small amount of yellow impurity was removed. The benzene was evaporated from the eluate. The remaining yellow material was crystallized from acetone. The recovery was 0.68 g. or 68% of the theoretical; m. p. 128°. Radioactivity assay: 0.130 mc./mole.

Attempted Exchange of Benzaldehyde with β-Nitrostyrene - A 0.65-g. sample of β-nitrostyrene and 0.2 g. of sodium hydroxide were dissolved in 15 ml. of methanol, and 2 ml. of water were added. To the cooled solution was added 0.5 g. of benzaldehyde sodium bisulfite compound (2.320 mc./mole). The mixture was stirred for 12 hours at room temperature. The mixture was acidified, diluted with water and extracted with ether. The ether solution was dried. When the ether evaporated, a red oil remained which did not crystallize after several days in the cold. The substance would not crystallize from any of a number of solvents tried. Since the oil could not be characterized, it was not assayed for radioactivity. The Geiger-Muller counter showed that the oil contained some radioactivity.

Attempted Exchange Reactions with Cinnamic Acid - A 0.5-ml. sample of radioactive benzaldehyde was added to a mixture of 0.7 g. of cinnamic acid, 0.46 g. of dried potassium acetate, and 4 g. of diphenyl. The mixture was heated for 6 hours at 170°. After the mixture had cooled, ether and dilute hydrochloric acid were added. After the mixture was thoroughly shaken, the ether layer was separated and washed with dilute hydrochloric acid and water. The cinnamic acid was extracted from the ether with saturated sodium bicarbonate solution. When this solution was acidified, the acid was removed by filtration and redissolved in ether. This solution was washed with water and extracted with sodium bicarbonate solution. After acidification, the product weighed 0.57 g. or 74% of the theoretical recovery; m. p. 130°. The acid was filtered off and dried. The Geiger-Muller counter showed that the cinnamic acid contained no radioactivity. Similar results were obtained when a mixture of cinnamic acid, acetic anhydride and sodium-2-$C^{14}$ acetate was heated.

The reaction conditions and the results of several experiments with cinnamic acid are compiled in Table VII.

## Preparation of Labeled Flavanone Utilizing Exchange Techniques

Preparation of 2'-Hydroxy(benzal-7-$C^{14}$)acetophenone - A 1.1-g. sample of 2'-hydroxybenzalacetophenone and 2 ml. of 20% sodium hydroxide were dissolved in 20 ml. of ethanol. To the cooled solution, 0.5 ml. of benzaldehyde-7-$C^{14}$(1.49 mc./mole) were added. The solution was stirred for

TABLE VII

Attempted Exchange Reactions of Cinnamic Acid

| Exp. No. | Grams Cinnamic Acid | Labeled Component | Grams of Labeled Component | Catalyst | Amount of Catalyst | Temperature | Hours of Reaction | Remarks** |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.9 | Acetic Anhydride | 0.62 | Sodium* Acetate-2-$C^{14}$ | 0.5 g. | 170-5° | 4 | |
| 2 | 0.9 | Acetic Anhydride | 0.62 | Sodium* Acetate-2-$C^{14}$ | 0.5 g. | 170° | 6 | |
| 3 | 0.35 | Benzaldehyde Sodium Bisulfite Compound | 0.5 | NaOH | 0.25 g. | 25° | 24 | 2 ml. of water and 30 ml of ethanol added. |
| 4 | 0.5 | Benzaldehyde | 0.25 | Piperidine | 3 drops | 170° | 6 | |
| 5 | 0.7 | Benzaldehyde | 0.5 | Potassium Acetate | 0.46 g. | 170° | 6 | 4 g. of biphenyl added as solvent. |

* The sodium acetate-2-$C^{14}$ was kindly supplied by Mr. V. F. Raaen, Oak Ridge National Laboratory. It was assumed that the sodium acetate would exchange completely with the acetic anhydride [36], the actual reacting species in the normal Perkin reaction.

** In none of these experiments was exchange obtained.

60 hours at room temperature. After dilution with water and acidification, the crude product was removed and crystallized twice from methanol. After drying, the product weighed 0.98 g. or 89% of the theoretical amount; m. p. 86° [91]. Radioactivity assay: 0.130 mc/mole.

Preparation of Flavanone-2-$C^{14}$ - A 0.5-g. sample of the 2'-hydroxybenzal-acetophenone was dissolved in 30 ml. of methanol and 1 ml. of a 1% solution of sodium hydroxide was added. The mixture was stirred for 24 hours at room temperature. The pH of the solution was adjusted to 6 with hydrochloric acid and 1 ml. of water was added. On cooling, the flavanone crystallized. After 5 crystallizations from methanol and drying, the product weighed 0.4 g. which corresponds to 80% of the theoretical yield; m. p. 74° [91].

# CHAPTER IV

# DISCUSSION

Paper Chromatography of Flavonoid Compounds - With the publication of the work of Bate-Smith [6] and Wender and Gage [7,92], standard $R_f$ values for some of the flavonoid compounds in certain solvent systems became available. The work reported in this thesis extends this work to additional compounds and solvent systems. The four systems of relatively high water-content, namely, 60% and 15% acetic acid or 22% and 60% isopropyl alcohol were found to be particularly useful. The pigment bands which resulted were sharply defined, and the $R_f$ values were highly reproducible.

It is now possible to classify a given flavonoid pigment into one of the major groups listed in Table II by utilization of the information and techniques described in this thesis and in the thesis of Gage . As an example, consider that a pigment is observed to fluoresce with a brownish-yellow color in ultraviolet light and to react with basic lead acetate to produce a yellow color in visible and a bright orange in ultraviolet light. Its $R_f$ value is observed to be in the intermediate range in the solvents of high water content. This pigment may be tentatively classified as a flavonol glycoside. If a hypothetical pigment is observed to fluoresce with a brilliant yellow color in ultraviolet light and to move at a low rate in solvent systems of high water content, it is likely to be a flavonol aglycone. It is possible to draw other generalizations concerning the other classes of pigments from a knowledge of $R_f$ values and the colors produced by the various chromogenic reagents.

During the search for new solvent systems suitable for the flavonoid compounds, several combinations of solvents which were tried

contained either pyridine or collidine. It was found that these substances caused the pigments to yield ill-defined streaks on the chromatograms instead of sharp bands. Discoloration of the pigments was also observed, indicating that decomposition had occurred.

Two-dimensional paper chromatography has not proved particularly successful for flavonoid compounds, because of the rather large zones formed during the second development. It should be possible to find solvent systems in which these compounds produce sufficiently sharp bands to make this method useful. In using one-dimensional paper chromatography in examining concentrates from natural products, it became very difficult to correlate the $R_f$ values obtained in a given solvent system with those obtained in another, particularly if the components of a mixture of compounds were similar in structure. The use of two dimensional chromatography would simplify the analysis for the pigments, since it would make possible the determination of two $R_f$ values for a given pigment without ambiguity.

The procedure of the hydrolysis of the glycosides on the paper would be particularly useful when applied in conjunction with two-dimensional chromatography. After the naturally occurring mixture had been separated by the first development, the glycoside could be hydrolyzed and the chromatogram developed in the second direction. In this manner, the $R_f$ values of the glycoside, its aglycone, and its sugar could be obtained. It should be noted that complete reaction was not always obtained by the hydrolysis procedure described. Frequently some of the unhydrolyzed glycoside was found after development.

The use of Benedict's solution in the location of zones on developed chromatograms has not been previously reported. When the solution was sprayed on a chromatogram containing a flavonoid compound, a bright spot, usually yellow, appeared immediately. While the color developed is probably due to the base present in the solution, the blue background caused by the copper sulfate made the test very sensitive. The yellow spot produced by a very small amount of the pigment was made much more easily visible by its contrast with the blue background.

The location of sugar zones on chromatograms was also possible with Benedict's solution. Two methods for the production of the cuprous oxide on the paper were tried. In the first, the sprayed chromatogram was suspended over a large beaker of boiling water for ten minutes. Although the reduction was successful under these conditions, difficulty was experienced in handling the wet chromatogram. It was found that if the freshly sprayed chromatogram was placed in the oven at 100° for ten minutes, orange spots were produced, and the further handling of the paper strips was facilitated.

Partridge[81] has reported the development of a yellow color when the "aniline hydrogen phthalate" reagent is sprayed on certain carbohydrate zones on chromatograms. This was confirmed with glucose and rhamnose. It was found in the research reported here, however, that the test was made much more sensitive by examining the sprayed chromatogram in ultraviolet light. Very distinct white fluorescent spots appeared at the sugar zones.

Chromatographic Isolation Procedures - The work reported in the experimental section under this heading is by no means a complete survey

of the field. Two significant developments are noted, however. By using filter paper pulp as an adsorption medium and by proper choice of solvents, it should be possible to attain separations somewhat analogous to those obtained in paper strip chromatography and the relatively large-scale fractionation of naturally occurring mixtures of flavonoid compounds would become a much simpler procedure than it is at present.

The use of starch columns in the purification of flavonoid fractions offers great possibilities. If all or most of the foreign material is removed, the paper chromatographic identification of the compounds of the mixture is facilitated a great deal.

"Dow Methocel" does not appear to offer much hope as an adsorbent because of its great tendency to form gels in aqueous systems.

The development of the ion-exchange purification technique of Morris et. al.,[16] is novel and revolutionary in the isolation of flavonoid fractions from natural materials. The work on the various adsorption media reported in this thesis was directed toward finding a similar method.

<u>The Flavonoid Constituents of Big Bend Locoweed</u> - While the evidence is not conclusive, it appears that the compound which was isolated from the weed is probably isoquercitrin. The reported melting point of isoquercitrin is 217-219°[93]. The compound isolated melted at 200-205°. Since only 15 mg. of the material was obtained, extensive purification could not be undertaken; it is therefore possible that impurities were present. The presence of impurities was suggested by the ultraviolet adsorption spectrum. Isoquercitrin exhibits absorption maxima at 255 and 355-360 mμ, while the isolated material shows

maxima at 260 - 265 and 350 - 355 mμ. It will be noted that both bands appear to be shifted slightly and that the band in the region of the longer wave lengths is markedly depressed. This depression is great enough to indicate the presence of impurities. The results of the various color tests reported were identical with those observed with an authentic sample of isoquercitrin. Only glucosazone crystals were observed when the hydrolyzate of the isolated compound was treated with phenylhydrazine. On paper chromatography, the aglycone present in the hydrolyzate exhibited $R_f$ values very close to those of quercetin as reported by Wender and Gage [7]. The $R_f$ values of the glycoside were very close to those of isoquercitrin in water-saturated phenol and 40% butanol-50% water-10% acetic acid. The $R_f$ value of the glycoside in water-saturated ethyl acetate was 0.65. That of isoquercitrin is reported to be 0.40. Neither simultaneous nor mixed chromatograms of isoquercitrin and the isolated pigments were run. Both of these procedures should be carried out in attempts to establish the identity of a compound by paper chromatography. It should be mentioned that the duplication of $R_f$ values in solvents of relatively high volatility is often difficult.

Paper chromatography of the fraction from the weed containing the flavonoid compounds, isolated as described on page 39, showed that several pigments were present. When 60% acetic acid was used as the developing solvent, four bands were observed, and in 40% butanol-10% acetic acid-50% water, five bands appeared. The first band in 60% acetic acid was bright yellow when viewed in ultraviolet light and its $R_f$ value was 0.41. In the butanol-acetic acid-water system quercetin has an $R_f$ value of 0.78. On chromatography in this system, the flavonoid fraction exhibited a band with an $R_f$ value of 0.79. This band, however, was a brownish-yellow in

color. Isoquercitrin and quercimeritrin both had $R_f$ values of 0.72 and quercitrin 0.82 in this system, and all these compounds were brownish-yellow in the ultraviolet. It is possible that one of these compounds may have masked the quercitrin band in the butanol-acetic acid-water system. This assumption is supported by the fact that a brownish-yellow band with an $R_f$ value of 0.75 was observed in the 60% acetic acid system. Isoquercitrin and quercimeritrin have $R_f$ values of 0.73 and 0.74 respectively in this solvent. A band with bright yellow fluorescence in ultraviolet light and an $R_f$ value of 0.55 was found on chromatograms developed in 60% acetic acid. The bright yellow color suggested a flavonol aglycone. Several of the flavonol aglycones have shown $R_f$ values relatively close to this value. Kaemferol and patuletin both have shown $R_f$ values of 0.50 in this system, while nortangeretin has a value of 0.54 and rhamnetin one of 0.60. A band of the same color with an $R_f$ value of 0.85 was observed when the butanol-acetic acid-water system was used. Of the aglycones mentioned above only two have values close to this. Rhamnetin has a value of 0.80 and kaemferol a value of 0.85. It is possible that one of these compounds occurs in the weed.

Bands of pale yellow in the ultraviolet and having $R_f$ values of 0.98 and 0.86 respectively were observed in the butanol-acetic acid-water and 60% acetic acid systems. The pale yellow color of this substance suggests that it is a flavanone. It is not possible to speculate as to the identity of this compound, since the $R_f$ values of only five flavanones have been determined.

The band having an $R_f$ value of 0.50 - 0.54 in the butanol-acetic acid-water system was brownish-yellow when viewed in ultraviolet light. The $R_f$ values of this compound fall fairly close to those of rutin, robinin, and xanthorhamnin. The $R_f$ values of these three pigments in the butanol-acetic acid-water system are as follows: rutin, 0.57; robinin, 0.51; and xanthorhamnin, 0.50. The color of robinin in ultraviolet light is a much more definite orange than the other two compounds thus possibly eliminating it from further consideration. Rutin and xanthorhamnin have $R_f$ values of 0.75 and 0.82 in 60% acetic acid. The band with the $R_f$ value of 0.75 in this solvent discussed above in connection with isoquercitrin and quercitrin might possibly be one of these compounds.

In the butanol-acetic acid-water system a band was observed which cannot be related to any of the compounds which have been studied by chromatography. It was bright yellow in color when observed in ultraviolet light and had an $R_f$ value of 0.38.

The statements made in this section as to the identity of the various flavonoid compounds present in the locoweed are to be considered only as tentative possibilities, since further work is necessary before more definite statements may be made.

The difficulty of correlating $R_f$ values obtained in one solvent with those observed in another in one-dimensional paper chromatography is well illustrated in the work reported here. Had it been possible to use two-dimensional chromatography, much more definite information as to the identity of the compounds would have been obtained.

The Flavonoid Constitutents of Thompson White Seedless Grapes - In the procedure for concentrating the flavonoid pigments of the grapes without the use of ion-exchange resins, it will be noted that only the solid portions of the grapes were used. It is quite probable that the major portion of the pigments was not studied since the juice was not examined for its pigment content. In any comprehensive study of the flavonoid compounds from the standpoint of nutrition, it would be necessary to analyze the liquid portion also.

Two flavonoid compounds were observed on paper chromatograms of the solution which was obtained from the concentration of the compounds without using ion-exchange resins. The $R_f$ values of these compounds agreed very closely with those of rutin and quercetin. After partition chromatography of this solution on starch, however, three pigments were found on paper chromatograms. From its $R_f$ values the third pigment appears to be either quercimeritrin or isoquercitrin. The explanation for the appearance of this compound might be that it occurs in very small concentration and was masked by other materials present, and that the starch chromatography removed the masking impurity.

Since the concentration of sugars in the grapes is relatively high, the isolation of any other compound is made difficult by the fact that sugars are carried along through many purification procedures. The use of the ion exchange resins in the concentration of the pigments present in grapes was particularly efficient, since it was possible to separate all the sugars present from flavonoid compounds in one step.

After the compounds had been adsorbed from the extract of the grapes, it was found that more of the pigments could be obtained from the effluent from the columns by passing it over the resin in the potassium form. It appears that this is because the potassium resin was a more efficient adsorbent for the flavonoids and not that there was selective adsorption of the pigments on either form of the resin.

The analysis of the fractions obtained from the treatment of the grape extracts would furnish useful information as to the identity of the pigments present in the total grape.

The Flavonoid Constituents of Tokay Grapes - The same considerations concerning the portions of the grape investigation apply to the Tokay grapes as applied to the Thompson variety.

Using ordinary methods of isolation, a fraction was separated from these grapes which contained three flavonoid compounds. One of these compounds appeared yellow when the developed chromatograms were viewed in ultraviolet light. This material had $R_f$ values very close to quercetin in four different solvent systems. Two brownish-yellow bands were observed which reacted with basic lead acetate solution to give bright yellow bands in the visible and orange bands in the ultraviolet. These facts suggested that the compounds were flavonol glycosides. If the $R_f$ values tabulated on page 44 for the brown bands are arranged so that lower values are assigned to one of the substances, these values agree very closely with those of isoquercitrin. It is not possible to correlate the other value with any of the glycosides studied in this work. In this tentative identification it will be noted that

in this instance the assumption is again made that the $R_f$ value of a band of a given type can be correlated with the values of a band of the same type obtained in a different solvent.

The fractions "RW" and "RE" isolated from the grapes were examined by paper chromatography. Only one compound appeared to be present in fraction "RW". Although the high solubility of this compound in water suggested that it might be xanthorhamnin, the $R_f$ values for four different solvents did not confirm this. In 60% acetic acid and water-saturated phenol the values lay relatively close to those of xanthorhamnin. For 15% acetic acid and the butanol-acetic acid-water systems the values were rather far removed. Recently Ice and Wender[94] have isolated a compound whose hydrolyzate contained only quercetin and glucose. While the structure of this compound has not been determined, its high water solubility and $R_f$ values in several solvents make it appear that this compound and the material isolated from fraction "RW" may be identical. A difference of 0.10 is noted in the $R_f$ values in the phenol water system.

The fraction "RE" exhibited three flavonoid bands when chromatographed in butanol-acetic acid-water and water-saturated phenol, and gave two bands in 60% and 15% acetic acid. If the values obtained from the water-saturated phenol are disregarded for the moment, the other values compare fairly well with those of rutin, quercitrin, and isoquercitrin. The determination of the $R_f$ values in all the solvents, phenol in particular, should be repeated and mixed chromatograms should be run.

The fact that only one aglycone was detected in the hydrolyzate of the fraction in question supports this tentative identification. The $R_f$ values of the aglycone found in the hydrolyzate resembled those of quercetin very closely in three solvents.

The 2,4-Dinitrophenylhydrazones of Flavanones - The preparation of the 2,4-dinitrophenylhydrazones of several flavanones represents the first of a projected series of studies with the purpose of preparing and characterizing new derivatives of the flavonoid compounds.

The compounds offer certain definite advantages and disadvantages for identification. The most outstanding advantage is the formation of distinctive crystals. Pictures of the crystals of the 2,4-dinitrophenylhydrazone of naringin and homeriodictyol are shown in Figure 7. The ease of preparation of these compounds is illustrated by the formation of the 2,4-dinitrophenylhydrazone of naringin which is obtained in about 10 minutes at room temperature. The relatively high melting points of the compounds constitute a disadvantage in the identification of flavanones.

The compounds were subjected to paper chromatography in all of the solvents which have been used for the flavonoids. None of these solvents, however, caused movement of the original spots. The only solvent which was found to move some of the derivatives at an appreciable rate was 50% water-30% dioxane-20% acetic acid.

The characteristics of the ultraviolet absorption spectra of the flavanones were found absent in the spectra of the respective dinitrophenylhydrazones. The three spectra which were determined are very similar.

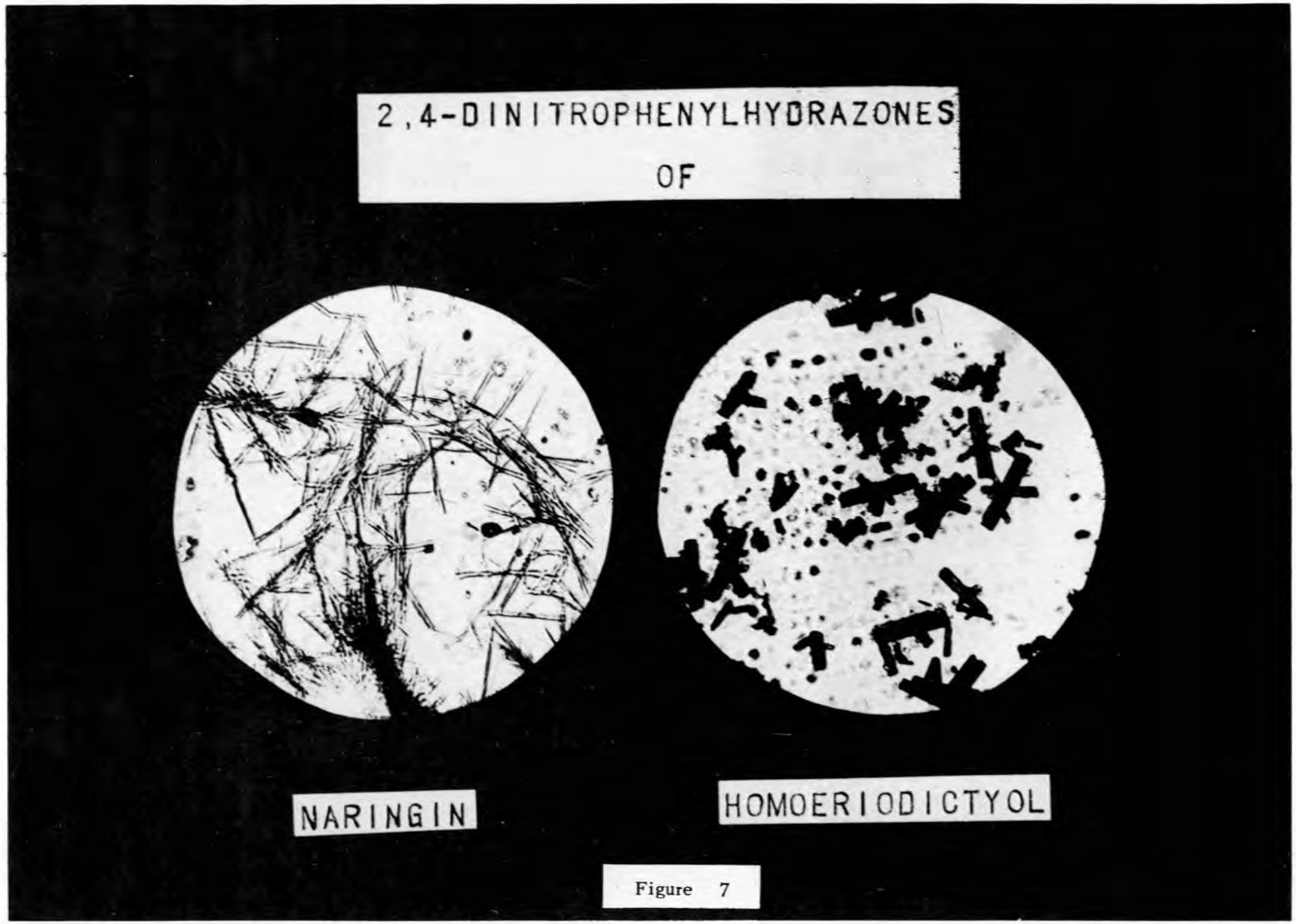

2,4-DINITROPHENYLHYDRAZONES
OF
NARINGIN
HOMOERIODICTYOL

Figure 7

Attempted Exchange in the Friedel-Crafts Acylation Reaction - In six experiments in which the solvent, temperature, catalyst, calalyst amount, and reaction time were varied, no set of conditions was found which promoted the exchange of acetyl-1-$C^{14}$ chloride with acetophenone. In four experiments, in which the conditions of reaction were varied, no exchange was found between benzophenone and benzoyl-7-$C^{14}$ chloride. A number of variously substituted acetophenones and benzophenones failed to exchange with the corresponding acyl chlorides under several sets of reaction conditions. These experiments clearly demonstrated the absence of an equilibrium between the starting materials and the final products in the Friedel-Crafts acylation reaction. The work of Olivier,[52] who was unable to find evidence of an equilibrium under one set of reaction conditions, was therefore substantiated.

Two processes may be visualized by which an atom or radical, B*, could exchange with the compound, AB. The first of these might be termed an $S_{N_1}$ process and this would be promoted by any electronic or steric effect which would loosen the bond between A and B:

$$AB \rightleftharpoons A + B$$

$$A + B^* \rightleftharpoons AB^*$$

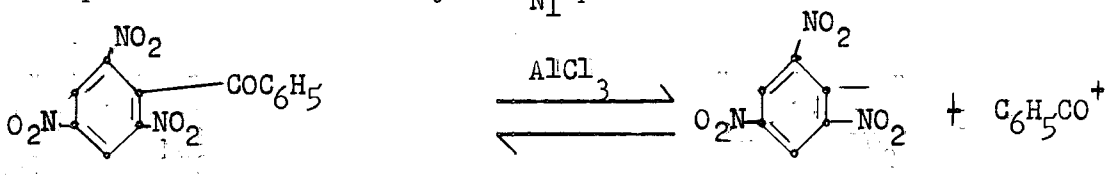
The second mode of exchange may be pictured as occurring through the reaction intermediate B*AB, by an $S_{N_2}$ process:

$$B^* + AB \rightleftharpoons B^*AB \rightleftharpoons AB^* + B$$

The governing step here appears to be the bond formation between A and B*. Since any electronic or steric effect which would promote

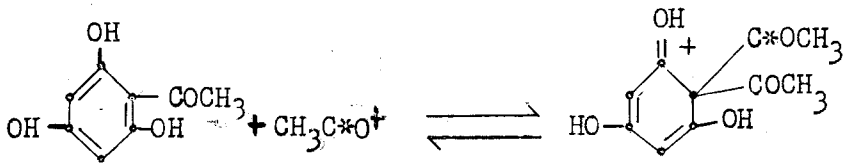
the formation of B*A would be expected to stabilize AB, those effects which lead to a more stable bond between A and B might be expected to promote exchange by an $S_{N2}$ process.

It seemed logical, therefore, that exchange of an acetyl or benzoyl group might occur with an aromatic ketone in which the ring contained strong electron-attracting or strong electron-repelling groups. Trinitrobenzophenone with its three electron attracting groups on one ring might be expected to dissociate by an $S_{N1}$ process.




That no exchange occurred with benzoyl-7-$C^{14}$ chloride, indicated that such activation did not promote exchange.

The converse of the above described system appears to exist with 2,4,6-trihydroxyacetophenone or 2,4-dihydroxyacetophenone. These compounds which have respectively 2 and 3 electron-repelling groups in one ring, might be expected to exchange with an acetyl group by an $S_{N2}$ process:




That such activation is insufficient was shown by the lack of exchange between the phenolic ketones and acetyl chloride.

Since the Friedel-Crafts alkylation has been demonstrated to be capable of reversal, the differences between this reaction and the acylation reaction will be examined. First, there appears to be a difference in the heats of the reactions. The alkylation reactions are usually carried out at room temperature. The acylations are usually cooled to prevent a violent reaction. These reactions are usually quite vigorous. Secondly, a catalytic amount of aluminum chloride is used in the alkylations as compared with a full molar proportion for the acylation reactions. This suggests that the reaction complex is probably transient in the case of the alkylation reactions. In the acylation reactions, the reaction complex is stable and usually precipitates from solution in a form which must be hydrolyzed to obtain the final product. The question arises as to whether the complex formed from the reaction is the same as that formed by treatment of the ketone with aluminum chloride. It is possible that they are quite different in structure and reactivity. If the assumption is made that they are identical, then the great stability of this complex may be the controlling factor in preventing the exchange with an acid chloride. If the assumption is made that the complexes are not identical, then it may be that the necessary reaction complex cannot be attained by reaction of the ketone with aluminum chloride. It is possible that the reaction complex formed in the acetylation would exchange with acetyl chloride, although the ketone-aluminum chloride complex would not. An interesting series of experiments which might shed some light on the nature of the complexes would be to prepare the two types of complexes mentioned above and to determine whether or not they will exchange their ketone components with the radioactive ketone and with labeled acid chloride.

A third possible explanation of the difference in the reversibility of the two reaction types is the difference in the ease of breaking a $CH_2$-C bond to form an alkyl carbonium ion as compared to the ease of breaking a CO-C bond to form an acyl carbonium ion. These energy requirements are impossible to evaluate but may be sufficiently different to explain the differences in the reactions.

Experimental difficulties arose with the hydroxylated ketones which were not encountered with the other compounds. For example, the acetylation of the hydroxyl groups with the acyl chloride competed with the exchange reaction. Secondly, the condensation of a second acyl group with the ring was possible. An example of these side-product forming reactions is found in Experiment 9 as described in Table IV. From the attempted exchange of acetyl chloride with 2,4-dihydroxyacetophenone was obtained a compound containing radioactivity. From its melting point, solubilities, and one of its paper chromatograms it appeared to be identical with 2,4-dihydroxyacetophenone. Its mixed melting point with 2,4-dihydroxyacetophenone, its ultraviolet and infrared absorption spectra and its paper chromatogram in 50% acetic acid indicated that it was a different compound. Although on vigorous acid hydrolysis it was converted to 2,4-dihydroxyacetophenone, its melting point and other characteristics corresponded with no simple mono- or polyacetylated derivative or any simple diketone. Since the 2,4-dihydroxyacetophenone obtained by hydrolysis contained no radioactivity and hence no exchange had occurred in its formation, further identification work was not pursued.

It appears from these results that, in general, the Friedel-Crafts acylation reaction does not involve an equilibrium process. This does not imply that there may not be certain examples of acylation reactions which are reversible. For example, it has been reported[95,96] that certain mesityl ketones cleave in the presence of strong acids. This suggests that these ketones might exchange their acyl groups.

Exchange Reactions in Carbonyl-Methylene Condensations - With benzaldehyde-7-$C^{14}$ as the exchange agent, five reactions which involve the condensation of the aldehyde group with an activated methylene position and elimination of water have been found to be reversible. The experimental method, similar in all cases, has involved placing radioactive benzaldehyde and the appropriate condensation product together under the catalytic conditions which would be used for making the condensation product.

Three of the reactions involve methylene groups activated by an adjacent carbonyl group, i. e., the exchange of benzaldehyde with benzalacetophenone, 2'-hydroxybenzalacetophenone, and dibenzalacetone. One of the reactions involves a methylene group adjacent to a substituted aromatic ring, i. e., the exchange of benzaldehyde with 2,4-dinitrostilbene. The last of the reaction types concerned a methylene group which was part of a 5-membered ring, i. e., the exchange of benzaldehyde with 8-phenylbenzofulvene.

Each of the reactions was carried out in the presence of a basic catalyst and each reaction mixture contained added water or potential water from a reaction such as the following:

$$KOH + CH_3OH \rightleftharpoons CH_3OK + H_2O$$

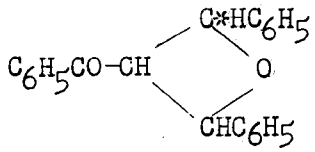
As indicated previously, condensations involving a carbonyl group and an active methylene position have usually been pictured as involving the reversible formation of the "aldol" product followed by the irreversible loss of water. With benzalacetophenone as the example, the following scheme illustrates the presumed mechanism.

$$C_6H_5COCH_3 + OH^- \rightleftharpoons C_6H_5COCH_2^- + H_2O$$

$$C_6H_5COCH_2^- + C_6H_5\overset{O}{\overset{\|}{C}}H \rightleftharpoons C_6H_5COCH_2CHOHC_6H_5$$

$$C_6H_5COCH_2CHOHC_6H_5 \longrightarrow C_6H_5COCH{=}CHC_6H_5 + H_2O$$

In order to rationalize the demonstrated exchange results with this simple picture, it is necessary either to assume complete reversibility of each step in this scheme or to demonstrate that exchange can take place by an independent path. The first alternative demands that the dehydration step be written as reversible such that water or an analogous reagent may add at the double bond. The second alternative would probably demand the formation of a symmetrically substituted intermediate with two benzaldehyde moities, e. g.:




Although sufficient data have not been accumulated to make a final decision, present evidence favors the former alternative.

Attempted exchanges between benzaldehyde and 2,4-dinitrostilbene were carried out under 3 different reaction conditions:

(1) In the presence of pure piperidine - no water or acid present,

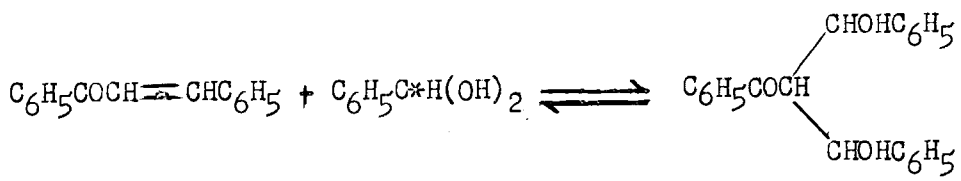
(2) In the presence of piperidine acetate,

(3) In the presence of piperidine acetate and water.

That exchange occurred only in the last case is evidence of the necessity of water. This is supported by the observation of Cope[70] that certain compounds formed in the Knoevenagel reaction hydrolyzed at the double bond by treatment with water at 125°.

The addition of water or an analogous reagent to the double bond is not surprising in view of the ease with which 2'-hydroxybenzalacetophenone isomerizes to form flavanone in either acid or basic solution. This reaction is in essence the addition of a phenol at the double bond.

The ease with which exchange occurs and the high recoveries suggest, however, the possibility of a "short-cut" route which does not involve complete reversal to the starting materials. The ease of exchange and the necessity of the presence of water could be explained by the formation of an intermediate analogous to that below:



In the absence of more experimental data, such a path must remain pure speculation. It seems probable that carefully designed exchange rate determinations as a function of the various reaction conditions might solve the problem.

The demonstration of the reversibility of the formation of 8-phenylbenzofulvene and of 2,4-dinitrostilbene are particularly interesting, since neither contains a carbonyl group. The addition of water at a double bond not conjugate to a carbonyl group would not be predicted.

An anomaly which arose in the preparation of the 8-phenylbenzofulvene has not been entirely removed. The observed melting point of 130° does not agree with that of Thiele [72]. He reported 88° as its melting point. Attempted bromination caused evolution of hydrogen bromide. It has not been possible to prepare a solid derivative. Since exchange with benzaldehyde-7-$C^{14}$ occurred and since it seems unlikely that the compound is other than 8-phenylbenzofulvene, the conclusion has been drawn that either the melting point given by Thiele is incorrect, or that the other geometrical isomer has been obtained.

In all the above experiments (except Experiment 3, Table XI ) the molar amounts of reagents were chosen which would yield a final theoretical molar activity ($A_{\infty}$) of one-half the initial activity of the benzaldehyde-7-$C^{14}$. In no case was the theoretical extent of exchange realized. This limitation may have been due to an insufficient time allowance for the reaction to go to completion. A second probable alternative is that certain irreversible side reactions, such as air oxidation, compete with the exchange reaction for the avail-

able benzaldehyde. These competing reactions should not be serious in the presence of acid catalysts.

Whether exchange will occur under acid catalysis has not been studied. It seems likely that acids will facilitate exchange in view of the report[68] that furfural may be displaced from its condensation products by other aldehydes in acid solution.

Since it is reported[97] that anhydrous aluminum chloride promotes certain carbonyl-methylene condensations, a particularly valuable experiment would be the attempted exchange in the presence of this catalyst. In view of the lack of exchange found in Friedel-Crafts acylation reactions reported previously, whether exchange would occur is problematical. Such an experiment might give an indication of the relative stability of aluminum chloride-carbonyl group complex addition products. Such data might be of value in evaluating the results of the attempted Friedel-Crafts exchange reactions.

No exchange was found to occur between benzaldehyde-7-$C^{14}$ and cinnamic acid under the influence of piperidine, sodium hydroxide, or potassium acetate at 180°. No exchange was found between cinnamic acid and acetic-2-$C^{14}$ anhydride at 180°. Although it is difficult to devise exchange reactions which exactly duplicate the conditions in the normal Perkin reaction, these data strongly suggest that this reaction involves no complete equilibrium.

The simplest explanation for the lack of reversibility of the Perkin reaction, in the absence of more complete data, is that water or an equivalent substance does not add at the $\alpha, \beta$ double bond of cinnamic acid.

Using benzalacetophenone as an example, the addition of water to the unsaturated system may be visualized as involving the initial addition of hydroxyl ion to the $\beta$- carbon atom:

$$C_6H_5COCH{=}CHC_6H_5 + OH^- \rightleftharpoons C_6H_5CO\bar{C}HCHOHC_6H_5$$

$$C_6H_5CO\bar{C}HCHOHC_6H_5 + H^+ \rightleftharpoons C_6H_5COCH_2CHOHC_6H_5$$

An alternative scheme would involve the addition of water to the $\beta$- carbon atom followed by rearrangement:

$$C_6H_5CO\bar{C}H - \overset{H\ H}{\overset{\diagdown\ \diagup}{\overset{O}{\overset{\|}{C}}}}H - C_6H_5 \longrightarrow C_6H_5COCH_2CHOHC_6H_5$$

In either case, the reaction involves a nucleophilic attack upon the $\beta$- carbon atom. Any electronic effect which increases the positive nature of the $\beta$- carbon atom would, therefore, be expected to facilitate attack by a nucleophilic reagent. Such attack would be expected to be aided by the normal polarizability of the 1,4 conjugate system:

$$C_6H_5\overset{O^-}{\overset{|}{C}}{=}CH{-}\overset{+}{C}HC_6H_5$$

That such an electronic shift is probably of much less importance in a compound with a carboxyl group instead of a carbonyl group could account for the lack of addition of water to the cinnamic acid unsaturated system.

That cinnamalacetophenone did not exchange with benzaldehyde-7-$C^{14}$ is surprising. Since cinnamalacetophenone is a vinylog of benzal-

acetophenone, such exchange might have been predicted to occur. It is possible that the positive charge endowed by the carbonyl group polarization is distributed over both the $\beta$ and $\delta$ carbon atoms:

$$C_6H_5\overset{O^-}{\overset{|}{C}} = CH - \overset{+}{C}HCH = CHC_6H_5; \quad C_6H_5\overset{O^-}{\overset{|}{C}} = CHCH = CH - \overset{+}{C}HC_6H_5$$

The net positive charge on each carbon atom would then be insufficient to attract the negative charge. This hypothesis could be evaluated by an experiment to determine whether cinnamaldehyde would exchange with cinnamalacetophenone.

Unfortunately, it was not possible to isolate the starting material from an attempted exchange reaction between benzaldehyde-7-$C^{14}$ and $\beta$ - nitrostyrene. Apparently an oxidation-reduction reaction occurred. It seems most likely that the condensation of benzaldehyde with nitromethane is reversible, since 2,4-dinitrostilbene, which might be considered a vinylog of the $\beta$- nitrostyrene, was shown to form reversibly.

<u>Preparation of Radioactive Flavanone Utilizing Exchange Techniques</u> - Since the experiments with exchange reactions have demonstrated the reversibility of the base-catalyzed condensation of benzaldehyde with acetophenone, the possibility exists that the low yields obtained in condensations with highly hydroxylated aldehydes and ketones might be due to an unfavorable equilibrium. Although this is not impossible in some cases, the fact that recoveries of condensation products isolated after exchange were always higher than the yields obtained when the com-

pounds are prepared, indicates that the equilibrium point is not the sole controlling factor. A reasonable explanation of the low preparative yields reported for the more highly hydroxylated benzolacetophenones may be the presence of competing irreversible side reactions. The reactivity of the carbonyl group of the benzaldehyde is undoubtedly repressed by the hydroxyl groups substituted in the ring. Competition with the ketone carbonyl group for the active methylene positions of the ketone and of the condensation product, i. e., a Michael reaction, becomes less favorable. These side reactions would be expected to be less serious in the exchange procedure. The ring closure of 2'-hydroxybenzalacetophenone to give flavanone was accomplished with aqueous base in 80% yield. It seems likely that this reaction is reversible also, since the pyrone ring may be re-opened by treatment with more concentrated alkali. It is possible that in the proper base concentration, benzaldehyde-7-$C^{14}$ could be made to exchange, through a number of steps, with flavanone directly. In view of the high yield of the ring-closure reaction, however, this technique seems unprofitable. The possibility of the condensation of the benzaldehyde with the hydrogen atoms at position 3 exists.

It is readily apparent that for the radiochemically efficient preparation of a labeled 2'-hydroxybenzalacetophenone, by exchange with radioactive benzaldehyde, the molar proportions of the two respective compounds in the exchange reaction should be at least 10:1. This proportion would give a theoretical radiochemical recovery in the condensation product of 90%.

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