A THESIS

entitled

"A Biochemical Investigation of the Distribution of Glucosides in Plants Indigenous to Northwest Canada and adjacent territories".

presented to

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by

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PART ONE

(1) Introduction.

(a) Present status of our knowledge of glucosides.

In the year 1901 Emile Bourquelot introduced enzymes into the laboratory as reagents(1). This marked an epoch in the study of glucosides and their distribution in plants, as previous to that time no systematic work had been done on these substances although ten glucosides had been discovered in a more or less accidental manner, e.g. salicin(2). The method of studying glucosides and their distribution devised by this noted savant has since become known as "The Biochemical Method of Bourquelot". The efficacy of the method is shown by the fact that out of 281 species of phanerogames examined in his laboratory, previous to the year 1920, 205 were found to contain one or more beta-glucosides(3). The method has been used by many French workers to demonstrate the presence of known glucosides in a large number of new plants, and to indicate the presence of previously unknown glucosides in a number of plants. This led to the isolation of a number of new glucosides.

(b) The principle of the "Biochemical Method".

The "Biochemical Method of Bourquelot" is based on the principle of enzymolytic hydrolysis. The distribution of sucrose is studied by the use of invertase; that of beta-glucosides by means of emulsin; and that of glucosides not

effected by the enzymes mentioned above by the use of "fermenting powders" prepared from the plants under consideration. The effect of these enzymes on plant extracts is measured by observing the changes, during hydrolysis, in the optical activity and reducing power. The result is qualitative, in that the ratio between the change in the reducing power and the change in the optical activity is specific for each glucoside; and the result is also quantitative, in that the total amount of the reducing sugar formed by the complete hydrolysis in an extract of known concentration gives the measurement of the glucosides present.

(c) Aim of the present work.

In the present work this method has been applied to a number of plants indigenous to the northwest part of this continent. The choice was only made after a phylogenetic consideration of those species found to be positive by the French workers. Thus it is purposed:-

1. To discover whether or not plants on this continent have the same glucosidic content as related species in Europe, and

11. To demonstrate the possible presence of new glucosides as a preliminary step towards their isolation and detailed investigation. These are all of potential pharmacological value in that they usually represent some of the most active compounds of the plant, i.e. hormones etc. The knowledge of

these compounds is of far reaching importance in the study of plant physiology. "The recognition of the potent effect of the constituents of glucosides in acting as stimuli and starters of active metabolism may be of importance in studying the nutrition of animals".(4)

(2) Detailed account of methods and technique.

(a) Preparation of the plant extract.

The fresh plant tissue is placed in a proportionate quantity of boiling 85% alcohol in a reflux apparatus, boiled for thirty minutes, filtered, minced, and againextracted in the same manner. The combined extracts are evaporated under reduced pressure, and taken up with sufficient thymol water to make one cubic centimeter of the extract represent one gram of the fresh material.

(b) Biochemical hydrolysis.

Invertase is added to two of three flasks each containing 25cc. of the extract. After incubation for one week at 37 C. emulsin is added to one of the flasks containing the invertase and incubation is continued for another week. The result is, one control flask, one flask in which the sucrose has been hydrolysed, and one flask in which the sucrose and beta glucosides (if present) have been hydrolysed. In that the emulsin of almonds usually contains invertase, it is necessary to hydrolysemwith invertase first even in those cases where only the beta-glucosides are to be studied.

(c) Clarification.

General method: A saturated solution of neutral lead acetate is added to each flask until no more precipitate is formed, and brought to equal volume with water. The precipitate is filtered off, the excess lead precipitated with hydrogen sulphide and removed by filtration. Aliquot quantities of the filtrate are neutralised with a concentrated solution of ammonium hydroxide, made up to equal volume with water, treated with animal charcoal by boiling under a reflux and filtered.

Frequently this is insufficient and special treatment must be devised to bring about satisfactory clarification.

(d) Estimation of the optical activity.

Three drops of ammonium hydroxide are added to each flask to stabilize any sugars and glucosides present. As shown by H.Colin and A.Chaudin (5) this is very essential in that the addition of NH_4OH during the reaction rapidly increases the rotatory power of beta-glucosides and reduces that of alpha-glucosides to the limit of rotation. After these changes are complete the optical activity of each is measured and expressed as degrees (with l=2 dcm.)

The first part of the work herein reported was done with a saccharimeter (F.Schmidt and Haensch no.7765), which was only graduated to 0.1% glucose. In spite of careful standardization this portion of the work suffered much on account of the inaccuracy of this instrument. The latter

part of the work was done with a "Duboscq-Pellin Precision Model" polarimeter of great accuracy.

(e) Estimation of reducing power.

The method of Benedict(6) was used exclusively in preferance to that of Bertrand (7),used by most of the French workers. The latter method is more tedious,requires a larger amount of solution than is available when the reducing power is low,and gives identical results. The former method, however,requires an absolute constancy of technique inorder to attain the same accuracy.

The reducing power is expressed as percentage glucose.

(f) The enzymes and their preparation.

As will be readily understood it is essential that the invertase used in the above proceedure be entirely free from beta-glucosidase. If this is not the case the addition of emulsin will bring about no further change and the method is valueless for the estimation of beta-glucosides. The invertase in the animal intestine is present in such small quantities that the preparation of a large amount was found to be very tedious. Yeast is the best source of invertase,so for this purpose a strain must be found that contains no beta-glucosidase. Top fermenting yeast from Shea's Brewery Ltd. reached this requirement,and was the only satisfactory strain of three tried. The method used by Bourquelot (8) to prepare

the invertase was found to be unsatisfactory, as all the enzymes and co-enzymes necessary for alcoholic fermentation are present, with the result that much of the sugar is destroyed before the necessary estimations can be made. The method of C.S.Hudson (9) consists of cytolysing by means of toluene and distilled water, precipitation of some impurities with lead acetate, removal of lead with hydrogen sulphide, and removal of acetic acid by dialysis. This is very satisfactory except that a solid preparation is more convenient in that it obviates change in volume. Such a solid preparation is very readily obtained by precipitating Hudsons solution with 2.5 volumes of 95% alcohol; decanting off the supernatant liquid after 16 hours; filtering or centrifuging; washing thoroughly with alcohol and ether to remove all traces of water; and drying in vacuo. If not free from water the precipitate, on coming in contact with the air, becomes dark brown instead of remaining perfectly white. This preparation has the advantage over all commercial preparations seen by the author in that it gives no color to an aqueous solution, and thus precludes the necessity of clarifying a solution which is otherwise clear.

The emulsin was prepared, by the method of Bridel and Arnold (10), from almonds and was entirely satisfactory.

The preparation of invertase is extremely active, 50 mg. being sufficient to change the optical activity of 25cc.

of an 8% sucrose solution to zero in ninety minutes. There is no perceptible loss in activity within eight months. The emulsin is also quite active; 62 mg. will change ten cc. of a 5% solution of amygdalin to a rotation of zero in two hours.

(3) The enzymolytic reduction index.

The ratio $\frac{\text{increase in reducing power(measured as mg.glucose)}}{\text{change in optical activity(degrees with l=2 dcm.)}}$ is known as the enzymolytic reduction index, and is specific for each pure glucoside. This has been calculated for sucrose and a large number of beta-glucosides by the French chemists. In using this index when working on extracts of plant tissues it must be remembered that if one obtains the index for a known glucoside it does not necessarily signify the presence of that glucoside, and vice versa, as it may be complicated by a number of factors. For example there may be two or more glucosides present and the combined indices give the result obtained. Also in the estimation of the optical activity of the original solution the glucoside itself may not be present, having been removed by the process of clarification, and unless the index for the pure glucoside has been obtained after the same process of clarification the indices will not be comparable. On the other hand if two closely related plants give the same index it can be assumed with a moderate degree of safety that their glucosidic content is the same.

PART TWO

(1) General account of work done.

During the summer of 1925 fifteen species of plants were collected in sufficient quantity for preliminary survey, and the initial treatment carried to a stage at which the extracts could be preserved without decomposition. Estimations have been completed on certain of the plant tissues for twelve of these species: the remainder, including certain of the various tissues separately extracted in some of those species already studied, are now being examined.

(2) Detailed account of work done with discussion of results.

A. Assays in which the saccharimeter was used.

1. Salix interior--Rowlee. (willow)

This specimen was identified by Mr. J. F. Higham of the Manitoba Agricultural College; it was collected on June 17th and extracted on the following day.

It has been known for many years that the bark of different species of Salix contains a beta-glucoside, viz. salicin(2). The bark of Salix interior is shown to contain this substance by the following results.

	Optical Activity	Reducing Power	Index.
After invertase.	+0.31°	0.710%	
After emulsin.	+0.65°	0.830%	353

In that the index for salicin is 321, these results are plainly indicative of its presence.

2. Aralia nudicaulis L. (wild sarsaparilla)

This plant was collected on June 9th and identified by Mr. J. F. Higham. The following results were obtained from an extract of the roots made the day of collection.

	Optical Activity	Reducing Power	Index
Original.	+0.27°	1.176%	
After Invertase.	-0.01°	1.363%	667
After emulsin.	±0.00°	1.357%	---

The enzymolytic reduction index for sucrose is 603, which so approximates that found in the above determinations that the presence of that sugar is almost certain. There is no significant change after incubation with emulsin, which is the result anticipated, in that so many of the closely related species contain glucosides not hydrolysed by this enzyme. Hedera helix is known to contain the flavone glucoside rutin. Sarsaparilla of many varieties are known to contain saponins, e.g. Honduras sarsaparilla(11).

3. Asarum canadense,L. (wild ginger)

This collection was made at Victoria Beach on June 13th, identified by Mr. C. W. Lowe of the University of Manitoba, and extracted the same day. Estimations were made on the various parts of the plant with the following results:

Leaves.	Optical Activity	Reducing Power	Indices
Original.	+0.68°	0.287%	
After invertase.	+0.27°	0.625%	824
After emulsin.	+0.28°	0.740%	---

Stems.	Optical Activity	Reducing Power	Indices.
Original.	+0.40°	0.114%	
After invertase.	+0.26°	0.175%	435
After emulsin.	+0.21°	0.186%	---
Roots.			
Original.	+0.42° (?)	0.184%	
After invertase.	+0.27°	0.422%	---
After emulsin.	+0.27°	0.500%	---

In that there is a large experimental error, on account of the instrument, in measuring the optical activity the results of the hydrolysis by invertase are taken by the author to be a demonstration of the presence of sucrose in all the tissues examined. The changes in the reducing power after emulsin indicate the presence of a glucoside in every case. The accompanying changes in optical activity could not be demonstrated with the instrument in use, and were therefore probably rather small, which means that the index for the glucoside must be quite large.

4. Diervilla diervilla,(L.) MacM. (bush honeysuckle)

This plant was identified by Mr. C. W. Lowe on June 13th, collection and extraction was carried out on the same day.

Root; 1st assay	Optical Activity	Reducing Power	Indices
Original	+1.06°	0.280%	
After invertase.	+0.08°	0.741%	470
After emulsin.	+0.73°	1.250%	783

2nd assay.	Optical Activity	Reducing Power	Indices
Original.	+0.60°	0.523%	
After invertase.	+0.02°	0.833%	534
After emulsin.	+0.71°	1.316%	700
Stems:1st assay.			
Original	+0.41°	0.549%	
After invertase.	-0.06°	0.840%	831
After emulsin.	+0.21°	1.087%	914
2nd assay.			
Original	+0.32°	0.388%	
After invertase.	±0.00°	0.654%	619
After emulsin.	+0.22°	1.(?)%	

In every assay there is definite evidence of the presence of sucrose. The indices vary considerably from that of sucrose (603) but are all within the limit of experimental error. Hydrolysis with emulsin definitely proves the presence of a beta-glucoside in the roots and stems. The index does not correspond to that of any of the known glucosides and suggests the probable presence of a glucoside hitherto not isolated.

B. Assays for which the polarimeter was used.

1. Cypropedium parviflorum, Salisb. (Downey Lady's Slipper)

This plant was gathered near Birds' Hill, Manitoba, on June 19th, and extracted on the same day. It was identified by Miss Grace Cameron of the University of Manitoba.

The flowers, leaves and stems were separately examined. In the case of the flowers only 19 grams of the fresh material was available to make the necessary 50cc. of solution for the study of beta-glucosides alone. Thus 1cc. of the extract represents only 0.38 gms. of the fresh material instead of the orthodox 1.0 gms.

The results of the estimations on the flowers are:

	Optical Activity	Reducing Power
After invertase.	+0.06°	0.877%
After emulsin.	+0.62°	0.703%

These results indicate that the emulsin brought about a synthesis, which used some of the reducing sugar previously present. It is rather confounding in that the optical activity changed to the right. One would expect either the formation of some beta-glucoside or gentiobiose, but if this had been the case the rotation would have been changed to the left.

The following results were obtained from the leaves:

	Optical Activity	Reducing Power.	Indices.
Original.	+2.37°	0.574%	
After invertase.	−0.27°	2.415%	697
After emulsin.	+0.15°	2.415%	25

The enzymolytic reduction index obtained from the changes after the use of invertase indicates that the greater part of the substance hydrolysed by that enzyme is probably sucrose (index 603). The fact that it is higher than the index for the pure sugar probably indicates the presence of some such substance such as stachyose or raffinose (cf. results on stems of same species).