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Thesis
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A THESIS

Part 1.

entitled

THE HALOGEN CONTENT OF NORMAL AND PATHOLOGICAL MAMMALIAN TISSUES .

presented to

THE DEPARTMENT OF BIOCHEMISTRY
of
THE UNIVERSITY OF MANITOBA .

by

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as partial requirement for the degree of Master of Science.

April 1928

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PART ONE

(1) Introduction

a) Present State of our Knowledge.

Although it has been known, for a long time, that chlorine, and possibly the other halogens were normal constituents of animal and plant bodies, it is only recently that definite information has been available as to its distribution. Previous to 1910 little work of value had been done on any of the tissues with the exception of the body fluids such as blood and lymph. In the case of these satisfactory methods had been developed for their halogen analysis and normal values well established. However, in 1910, Magnus-Levy (1) performed a series of investigations on a man who had committed suicide, and a few investigations on the tissues of other animals. He used Bunge's method which was extremely laborious but probably quite accurate.

Since 1910 very little has been done except in the case of the body fluids and excretions which were easily obtained normal. This marked lack of work on an admittedly important subject was possibly due to the lack of a suitable accurate method, which would be simple enough to allow of the study of a large number of analyses. Highly involved proceedures such as that of Carius obviously prohibited the necessary great number of analyses. In 1923, Van Slyke (2) evolved a relatively simple and apparently accurate procedure, and it is his method which I have used in the following work.

Although Van Slyke's Proceedure gives results as total halogen content, I have expressed my results as Chlorine

in milligrams per hundred grams of tissue. My presumption, of course, being that practically all the halogen in the body is chlorine with the exception of the iodine contained in the Thyroid Gland.

b) Discussion of the method.

1. Van Slyke's method is essentially a modification of several previous methods. Briefly it consists of the digest--ion of a small weighed quantity of dried tissue, with a known quantity of Silver Nitrate in concentrated Nitric Acid, on a boiling water bath, for twelve to eighteen hours. After the digestion the resulting clear yellow fluid, containing the curds of precipitated Silver Halide, is titrated directly with standard Amonnium Thiocyanate solution, using solid Ferric Alum as an indicator.

2. Previous to the adoption of Van Slyke's procedure it was deemed necessary to take greater precautions for the destruction of the organic material, by means of combustions, or oxidation with a Permanganate or other strong oxidizers. However Lawrence and Harris (3) have shown, at least in plants, that concentrated Nitric Acid was sufficient to break down all coloring and other organic materials, if sufficient time was allowed for the digestions. These authors point out that some tissues such as blood take longer than others, due to the presence of some such substance as Haemaglobin, which resisted the Nitric Acid for a longer period. This has also been born out by previous work in this department (4). Secondly in all the former procedures the precipitated Silver Halide was always filtered off to prevent its possible reaction with the Ammonium

Thiocyanate, and thus causing abnormally high results. However in the Van Slyke proceedure the Silver Chloride is precitated in such large curds as to prevent its rapid reaction with the thiocyanate. From a consideration of the above it will be seen that this method overcome many of the difficulties met with before, without any loss in accuracy. It is worth noting that the chance of experimental error is materially lessened by the fact that the solutions are not transfered from one vessel to another and thus mechanical loss is minimized.

To further test the accuracy of the method I performed a series of blank estimations on distilled water, + 5 % solutions of cane sugar containing known quantities of Sodium chloride

Solution	2 hours	8 hours	12 hours
20 cc. dist'd water (macro)	0.000% Cl.	0.001 0.000	0.000 0.001
20 cc of 5% sugar Sol'n	0.005% 0.002	0.0000 0.000	)macro) (micro)
20 cc. of a 0.1% sol'n of KCl (Mac)	0.098% 0.099	0.098 0.098	0.099 0.099
	Macro	Micro	
5% Sugar sol'n (1.82% KCl)	1.822% 1.810	1.800 1.826	
5% Sugar Sol'n (o.15% KCl)	0.152% 0.152	0.149 0.160	

The accuracy of these results may be judged from the fact that one drop of the N/20 thiocyanate solution used in the macro estimation made a difference of 0.01% in the result.

As an additional check, 10 cc. of N/20 Silver Nitrate, Nitric Acid solution, (standard reagent), was placed in a conical flask, covered with a funnel, in the usual way, and left on the laboratory bench for for ten days. At the end of that period there was no visible precitate of Silver Halide, nor was any indicated in the subsequent titration. This indicates that no halide is taken up from the air during the period of digestion.

2 a. In order that the efficiency of this method might be comparable to others I ran a series of comparisons with a modification of Kendall's method for Iodine estimation. (5) Breefly the method consisted as follows. 0.5000 grams of dessicated tissue were placed in a nickel crucible and heated, on an electric plate, with 20 cc. of 30% Sodium Hydroxide (Note: This amount of Caustic contained 5.51 milligrams of chlorine). The heating was continued until all moisture was driven off and a paste formed. The crucible was then transfered to a larger nickel crucible over a Meeker burner and heated until all gas evolution had ceased, after adding small quantities of Potassium Nitrate. The hot liquid was then poured carefully onto a a nickel lid to cool. The cold cake was dissolved in about 200 cc. of water, by boiling (including hot washings from crucible). The solution was then boiled down to 100 cc. volume on an electric plate, allowed to cool and then acidified with concentrated Nitric Acid; 50 cc of Silver Nitrate solution, of which 1 cc. was equivalent to 0.5 mg. of chlorine., was added and the total volume made up to 250 cc.

The whole was filtered and aliquot portions, of 50 cc. each, were titrated with standardized Ammonium Thiocyanate in the usual way. The results follow in Table I.

Table I.
Comparison of Van Slyke's & Kendall's Methods
Tissues from dog No.4; Results in mg. Cl. per 100 gms.(Dried)

TISSUE	V.Slyke Proc.	Kendall Proc.	Diff.%
Gastric Mucosa	898 mg.cl.	980	10%
Heart	353 357	356	0.03%
Lung	1,037 1,028	996 973	1.7%
Liver	438 446	530	(poor sampling due to large lumps)
Kidney	1,260 1,278	1,274 1,068	0.4%
Muscle (vol.)	252 250	248 266	2.4%
Spleen	785 802	796 812	1.4%

Fairly good agreement is indicated in the above with the exception of Gastric Mucosa, of which extremely small quantities are available. The other differences are quite within the limit of experimental error when it is remembered that it was impossible to obtain chlorine free Sodium Hydroxide (Baker's & Merck's Purest). In each estimation at least 5.51 milligrams of chlorine were introduced , and as Sodium Hydroxide is so hygroscopic, it was almost impossible to estimate this error accurately. Also, due to the relative complexity of the Fusion procedure, there was a greater possibility of error creeping in in that method. I consider

that these results show that the method of Van Slyke gives the correct halogen content and that it is much the preferable method due to its relative simplicity.

3. Modification of the original procedure

Previous work in this department (4) had indicated that a two hour digestion period was sufficient for most tissues, other than blood or its various parts, instead of the twelve hour period recommended by Van Slyke. In all my work, reported below, I allowed the digestion to proceed for a two hour period, only. However I noticed in a series of investigations that an increase in the time of digestion seemed to indicate a higher result, as shown in the following;

Tissue	Am't used	2 hrs.	4	6.	8.	12.
Lung	1.0000 gm.	976 mg.Cl.			1,060	
Liver	1.0000	519 540			675 650	
Liver	0.7000	533	546 601	697 538	611 670	604 529

The above tissue was obtained from Dog no. 5 (see following). Obviously the above results indicated some source of error other than technical. The possibility of absorption of halide from the air, during the longer digestion period seemed to be negatived by the observation, recorded above, where a solution was left on the bench for a period of ten days, with no apparent change. It was suggested that the silver may have combined with a Purine which would be liberated only after a long digestion. In view of this idea I performed a series of investigations on a Commercial preperation of Nucleic Acid (British Drug Houses Ltd.)

Am't used	2 hrs.	4.	6.	8.	12.
0.8000 Gms.	459 mg. cl.	435	434	435	466
0.8000	464	456	----	424	455

Results in mg. chlorine per 100 grams of substance tested.
Obviously the idea of a Silver Purine had to be dropped,
as the time of digestion made no appreciable differnce in the
result. It was also suggested that the acid content beacme
lowered over the longer periods of digestion, thus altering
the titratable result. However even after twelve hours digest-
ion it was shown that over fifty percent of the acid remained.
Also the the addition of fresh acid at the end of digestion
did not alter the result appreciably, as shown;

	Am't used	2 hrs.	6.	8.	12.
With out HNO_3	1.0000 gm.	580 mg.	560	550	570
With add'n of HNO_3	1.0000	520	540	520	550

The only other possibility to suggest itself was that the
tissues were not uniformly sampled. This was particularily
probable in the case of the liver which was extremely difficult
to powder when dry. As blood contains a much higher content
of halogen than the various tissues (see below) it is quite
easy to see that the inclusion of a small amount of blood
would materially affect the result of the analysis of the
sample. Accordingly I powdered all the tissues to pass through
a no. 20 seive, and thus was reasonably sure of obtaining a
fairly uniform sample. The following results were obtained
from propely sampled tissue (Dog nO.5). Results are expressed
in milligrams of chlorine per hundred grams of dessictaed
tissue. Error;- 1 drop is equiv. to 10 mg. per 100 gms.

Tissue	2 hrs.	4.	6.	8.	12.
Kidney	1,386 mg 1.386	---- 1,386	1,386 1,393	1,395 1,391	1,404 1,396
Liver	540	530	560	540	550
Lung	970	980	970	960	970

I think that I am justified in assuming from the above that a two hour digestion period is sufficient for most of the tissues, with the exception of blood, and possibly fat which is not broken down very readily by concentrated nitric acid. This latter fact was noted in the case of analyses on substances containing a little fat.

Thus the only modification which I have adopted in my use of Van Slyke's procedure, is to use the two hour digestion period rather than that of twelve hours (1).

PART TWO

The WHITE RAT

The common White Rat was used in all the following analyses. In some cases the organs of two rats were combined, and in other cases the organs of only one rat were used.

The animals were starved for at least twenty-four hours (one exception) before dissection. They were anaesthetized by chloroform and dissected immediately. The fresh tissue was cleaned as thoroughly as possible and blotted with filter paper to remove any excess blood or other fluid, and then placed in weighed glass dishes and weighed to obtain their fresh weights. They were then placed in an oven for some seventy-two hours, at 103-105° C., weighed, and then heated in this oven again until a constant dry weight was obtained. (usually 4 to 5 days) This procedure was followed with dog and human tissue, also.

When thoroughly dry the tissues were properly powdered and set aside for analysis.

In the majority of these analyses the whole organ was taken, powdered and estimated in duplicate. The lymph glands were taken from the anterior triangle of the neck. In the case of the Salivary Glands the Sub-maxillary were always used. The bone was taken from the right Fibulo-tibia. In all cases it was found to be impossible to dissect the pancreas sufficiently fat free to warrant its analysis. The stomach and intestine were dissected out, slit open, and washed free of all debris and mucus, and the mucosae were then removed by scrapping with a scalpel. In all cases the rats were starved for twenty four hours before dissection, with the exception of the fifth which had been accidentally fed just previous to dissection.

Some difficulty was met with in the analysis of bone. A very poor end point was obtained which seemed to be due to a precipitation of the Ferric Alum by possibly phosphates. I attempted to overcome this by addition of a large excess of Ferric Alum, filtration, and subsequent titration. This may account for the relatively poor agreement obtained for bone tissue. (I did not experience this difficulty with the bone of dog or man.)

Although macro estimations were performed whereever possible, it was usually necessary to be content with micro estimations as there was seldom enough to do the former in duplicate. (Micro estimations were done when the quantity of material per estimation was less than 400 milligrams.) The results follow in Tables 2.& 3. and are expressed in milligrams of chlorine, per hundred grams of dried or fresh tissue, resp.

Table 2.

Halogen content in milligrams of chlorine per 100 gm. DRY tissue

Tissue	1.	2.	3.	4.	5.	6.	Average
Bone	M 163 mg	166	---	238	---	251	205
Brain	495	573	532	422	500	471	499
Heart	452	---	417	533	568	451	484
Intestinal Mucosa	419	279	398	465	---	429	398
Kidney	M 759	M 1,135	762	1,021	786	761	871
Liver	M 463	M 567	M 450	M 576	428	502	498
Lungs	976	970	927	853	834	896	909
Lymph Glds.	581	568	---	---	---	---	575
Muscle (Vol.)	M 234	M 232	M 238	M 248	224	221	233
Spleen	656	611	578	596	550	537	588
Stomach Mucosa	780	600	538	596	569	672	626
Salivary Glds	499	472	664	513	483	506	523
Testes	1,822	1,400	2,110	---	1,950	1,680	1,792
Ovaries				1,126			1,126

Note: M refers to cases in which the macro proceedure was used. In most cases the above figures represent the mean value from duplicate estimations.

The above figures are probably fairly accurate with the one exception of thet for the ovaries of rat no. 4. When dried they weighed only 16 milligrams, so that the expressed result can be only considered as indicating the approximate order of its halogen content. The details of the animals used in the above six series follow Table 3.

Table 3.

Halogen content in milligms. chlorine per 100 grams FRESH tissue

Tissue	1.	2.	3.	4.	5.	6.	Average
Bone	M. 103 mg	118	---	150	---	129	125
Brain	113	127	112	95	107	94	108
Heart	102	---	95	117	137	103	111
Intesinal Mucosa	88	83	78	94	---	79	84
Kidney	M 191	198	173	167	174	165	178
Liver	M 140	M 143	M 123	M 137	120	129	132
Lungs	217	210	196	177	188	188	196
Lymph Glands	168	143	---	----	---	----	156
Muscle (Volunt)	M 58	M 61	M 63	M 71	49	59	60
Ovaries	---	---	---	290	---	---	290
Salivary Glands	132	130	144	97	121	128	125
Spleen	149	139	136	---	122	127	134
Stomach Mucosa	166	145	118	121	120	147	136
Testes	231	187	246	---	244	203	222

Note: M refers to the macro proceedure.

The figures in Table three are calculated simply from the fresh and dried weights of the whole organs. In other words Table 3 is an adaptation of Table 2.

Details of animals in Tables 2 & 3.

Series
1. Organs of two male rats; Resp. wts. 310 & 290 grams.
2. Organs of one adult male rat; Wt. 254 gms.
3. " " " " " " " 278 "
4. " " " " female " " 175 " .
\#. (Uterus contained five well developed foetuses)
5. Organs of one young male rat, weighing 94 gms. combined with those of another weighing 107 gms., (The right kidney of the former was cystic and was accordingly discarded. These rats had, unfortunately been fed immediatly before the dissection.

6. Combined organs of two male rats weighing 95 & 94 gr ms, respectively.

Obviously the amount of blood in the tissues on dissection would materially affect the results since the halogen content of whole blood is materially higher than any of the tissues examined. However this was a f ctor which was almost impossible to control. (See below for a further consideration of this problem.)

The most striking results, of those given above, are those for the gonads, particularily the testes. On may explain the high value found in the lungs by their very rich blood supply, and that found in the kidneys by the fact that they are the sources of excretion of the halogens, but neither of these explanations are suitable in the case of the gonads. ~~Fe~~ I have summarized these results more fully, below. It is also noteworthy that the most constant result is found with voluntary muscle.

PART THREE

THE DOG.

In the following analyses seven different dogs were used, and the technique was similar to that used for the rat work, reported above. The dogs were anaesthetized with chloroform and dissected immediately. The same proceedure was followed with tissues as in the rats. The results for the first six dogs are given in Tables 4 & 5. The results for the seventh dog are given in a subsequent table, as this ~~was~~ dog was bled as thoroughly as possible by severing the carotid artery, under light anaesthesia, in an endeavour to show if there was any noticeable effect on the halogen content of the various tissues.

Dog — Table 4.

Total halogen content of the dogs' tissues, expressed in milligrams of chlorine, per 100 grams of dessicated tissue.

Tissue	i.	ii.	iii.	iv.	v.	vi.	Average
Adrenals	m 348	m 318	---	---	m 386	---	351
Bone	150	---	---	---	150	---	150
Brain	734	616	---	~~66-~~	667	749	692
Cartilage	---	---	---	---	437	503	470
Duodenal Mucosa	681	590	---	---	725	783	695
Heart	506	486	---	352	528	555	485
Kidney	904	956	---	1,269	1,220	1,386	1,147
Liver	---	509	---	441	512	542	501
Lung	1,050	976	---	1,073	1,013	973	1,009
Muscle	259	254	---	251	240	293	259
Ovaries	m 609	m 890	---	---	---	---	750
Pancreas	534	459	---	---	635	559	544
Salivary Glands	418	718	---	---	908	517	640
Spleen	729	656	---	794	726	724	726
Stomach Mucosa	1,199	1,291	---	898	1,085	1,124	1,149
Tendon	534	472	---	---	640	---	548
Testes	---	---	~~1,163~~ 912	~~1,152~~ ---	~~1,2-~~ 1,152	1,206	1,075
Thyroid	469	668	---	---	518	611	566
Spinal Cord	m 387	m 431	---	---	m 315	m 435	392
Uterus	m 1,107	m 878	---	---	---	---	993

Note: m refers to the micro procedure.

The detailed description of the animals used for the above analyses is given after Table 5.

Dog. Table 5.

Total halogen content of the dogs' tissues, expressed in milligrams chlorine, per 100 grams fresh material.

Tissue	i.	ii.	iii.	iv.	v.	vi.	Average
Adrenals	131	104	---	---	136	---	124
Bone	97	---	---	---	127	---	112
Brain	143	142	---	---	144	161	148
Cartilage	---	---	---	---	191	189	190
Duodenal Mucosa	144	100	---	---	132	148	124
Heart	119	129	---	93	124	128	118
Kidney	196	216	---	272	270	303	251
Liver	---	134	---	126	138	144	136
Lungs	217	228	---	242	230	232	230
Muscle	63	68	---	69	62	71	67
Ovaries	183	198	---	----	---	---	191
Pancreas	140	96	---	---	159	160	139
Salivary Glands	112	170	---	---	190	137	152
Spinal Cord	104	134	///	---	104	130	118
Spleen	165	188	---	180	164	158	171
Stomach Mucosa	205	143	---	127	143	183	160
Tendon	281	246	---	---	256	---	261
Testes	---	---	195	---	180	186	187
Thyroid	110	140	---	---	185	208	161
Uterus	198	204	---	----	---	---	201

The figures given in Table 5., are obviously derived from those in Table 4., from a consideration of the relative dry and fresh weights of the various tissues. It will be noted that apparent discrepancies in the dry weight table become agreeable in the second table.

In all cases, cartilage was taken from the Trachea; muscle from the adductors of the thigh (right); bone from the middle third of the right femur; tendon from the Tendo Achillis; the salivary glands used were always the Submaxillaries; the gastric and duodenal mucosae were prepared as in the case of the rat; the whole uterus, both horns, and cervix was used.

Details of animals exmained;

Dog 1. Young female, aged about four months. Wt. 3 Kilograms

2. Adult female dog. Weight 13 kilograms.

3. Adult male dog. Weight 22 kilograms. Only the testes of this dog were used. The animal was being used for experimental purposes in the Dept. of Physiology, and appeared to be normal in every way.

4. Adult female dog. Weight 6.5 kilograms. This animal was used for comparative analyses with the Fusion method of Kendall (See Introduction).

5. Adult male. Weight 21.6 Kilograms. Although starved its stomach contained a large amount of rope-like debris.

6. Adult male. Weight 19.5 kilograms. Gastro-intestinal tract was remarkably empty and clean.

7. Adult male. Weight 12.3 Kilograms. This dog was bled (see below)

Note: All the above animals appeared to be in good physical condition, although apparently starved for some considerable period. Their race was, of course, indeterminate.

These results agree very well with those obtained in rats with the possible exception of brain and duodenal mucosa which are somewhat higher in the dog. The remarkably high value for the gonads and uterus are extremely noticeable. Obviously the fresh weight figures are the only ones that are comparable.

As in thecase of rats it will be noticed that there seems to be quite a variation between the animals for the same tissue, and we thought that this might most likely be due to the amount of residual blood in the tissues on dissection. Accordingly I attemted to bleed the seventh dog compl tely by severing the right Carotid artery, under light anaesthesia.

The bleeding was carried out fairly succesfully, although it was impossible to make it complete by perfusion as this procedure would undoubtedly alter the halogen content of the tissues. Accordingly the dog died before all its blood could be drained. The balance of the procedure was the same as that used for the other dogs. Results in Table 6.

Table 6.

Comparison of halogen content of tissues of bled dog and average of other six(T.5) in mgm.chlorine per 100 gm Fresh material.

Tissue	Average of six	Bled Dog.
Adrenals	124 mg.cl.	113 mg. Cl.
Bone	112	101
Brain	148	154
Cartilage	190	172
Duodenal Mucosa	124	114
Heart	118	90
Kidney	251	243
Liver	136	107
Lungs	230	259
Muscle (voluntary)	67	69
Ovaries		
Pancreas	139	176
Salivary Glands	152	123
Spinal Cord	118	117
Spleen	171	175
Stomach Mucos	160	169
Tendon	261	225
Testes	187	188

Note: Titratable error in above would be 2 mgm. of chlorine in 100 grams fresh tissue, per drop.

Rememb ring that the halogen content of whole blood is much greater than the above tissues (360 mgm. cl. per 100 cc.) one might expect lower results in the bled dog. However the above comparison makes it clear that complete bleeding did not occur, and it seems to me impossible to do that. On might expect certain organs to lose their blood supply, earlier than others, and this might explain the

slightly lower results obtained in the heart, liver, salivary glands, and tendon. The high result in the case of the lungs might ~~hight~~ be due to a number of reasons any of which would cause an increased flow to those organs, such as increased respiration. In passing it might be noticed that the most constant results obtained for any tissue were those for voluntary muscle in both rat and dog. Working on the assumption that increaed blood supply would increase the halogen content one would expect marked variation in these figures. However I think a plausible explanation might be that the animals were all in the third stage of anaesthesia during the most of the dissection, and it is well known that all animals relax their muscles very much during this stage, and thus one might expect a diminution in their blood supply during that period. Similarily variable results might be explained in such tissues as the Salivery glands by the excitement of the animal or the general metabolic condition of the particular organ.

It has been suspected that the halogens may be organically combined, particularily in the stomach and Thyroid. That this is so in the latter is well established, but there is no evidence that they are combined in this manner in any other tissue. In order to demonstrate that all the halogens in the body are in inorganic form, with the one mentioned exception, I performed a series of estimations on water extracts of the tissues of dogs v & vi. These results are tabulated in Table 7.

Table 7.

Comparison of Van Slyke results and those from water extraction of tissues. Results in mgm Cl. per 100 gms dried tissue.

Tissue	Dog v. V.Slyke	Dog v. Water extract:	Dog vi. V.Slyke	Dog vi. Water extract
Bone			99	95
Brain			749	755
Cartilage			503	509
Duodenal Mucosa			783	743
Gastric Mucosa			1,124	1,115
Heart	528	515	555	518
Kidney	1,225	1,250	1,386	1,404
Liver	510	550	542	555
Lungs	1,015	990	973	965
Muscle	263	240	293	266
Pancreas	639	640	559	545
Salivary glds.	966	980	517	504
Spinal Cord			435	425
Spleen			724	720
Thyroid			611	561
Testes			1,152	1,149

Note: Titratable error is equivalent to 10 mgm per 100 grams dry tissue, per drop.

Water extraction method.

A weighed quantity of dessicated material (preferably 1.0000 grams) is placed in a large Erlenmeyer flask with about twenty (20) cc.s. of distilled water and boiled for about fifteen minutes (15). The extract is filtered by suction, and the residue washed twice with 20 cc. of boiling water. The filtered extract is allowed to cool and then 10 or 15 cc. of N/20 $AgNO_3$ in concentrated Nitric Acid is added. The whole is raised just to the boiling point, for a moment to precipitate any remaining colloidal material, and then the whole was allowed to cool. The excess silver nitrate is titrated against N/20 Ammonium Thiocyanate solution, using solid Ferric Alum, as an indicator. This procedure was followed with every tissue examined with the exception of bone, cartilage and tendon which I found required at least twenty-four hours continuous extraction on a boiling water bath, after raising to the boiling point as above.

From the above results I think it might be safely assumed that the halogens are not combined organically in the body, with the exception of the Thyroid gland which contains Iodothyroglobulin.

It is of interest to note that Roseman (5), 1910-11, who analyzed the whole body of dogs found that total halogen content varied from 112 to 120 milligrams of chlorine per 100 grams of fresh weight. Although one could hardly estimate the total halogen content from my results, it would appear that it would approximate that found by Rosemann. The latter also found that a high chlorine content, up to 136 milligrams, occured after several months of a high chlorine diet.

Magnus-Levy (1) published results for dog chlorine analysis taken from other authors, including Langlois & Richet, Wahlgren, Nenki, and Katz. However it seems that these results can hardly be taken as reliable. In Table 8 I have listed results obtained by Damiens (6) in 1921, along with my own.

Table 8.

Comparison of Damiens' results and my own, expressed in milligrams of chlorine per 100 grams of fresh tissue.

Tissue	Damiens 1.	Damiens 2.	Walton (average of 6 dogs)
Adrenals	220 mg.	122	124
Blood (Whole)	270		
Heart	160	111	118
Kidney	210	238	251
Liver	110	124	136
Lungs	210	270	230
Muscle	55	89	67
Testes	220	207	187
Trachea	80	190	190 (Cartilage only)

Note: Damiens 1. refers to a young dog and 2. to an old dog

Damiens method consisted briefly of a destruction of the organic material with alkaline carbonates and potassium nitrate, with subsequent solution and estimation with $AgNO_3$ in the usual manner.

As Damiens'method is very similar to the method of Kendall's which we adapted, it should be quite accurate and compare favourably with my figures. It will be noted that his figures compare very closely in every instance. Any slight variations probably being due to the various sources of error mentioned above. There is a slight suggestion that different results might be obtained due to the differnce in age of the animals examined, but in my analyses it will be noted that there are no marked differences between Dog 1. which was only a young puppy of about three months of age, do not materially differ from the results obtained from adult dogs of both sexes.(Tables 2 &3.).

Damion has shown in the same paper (6) that bromine occurs in the various tissues normally in a Br/Cl. ratio of .00081 to .00253. As Iodine is eldom found in the body, except in the thyroid,it follows that the majority of the halide present is in the form of chloride.

PART FOUR

MAN

In 1910, Magnus-Levy (1) made a detailed investigation of the halogen content of the tissues of a suicide victim. He used Bunge's method which, although laborious, was probably quite accurate. Since that time very little has been done on man. This fact is probably due to the difficulty of obtaining normal cadavers.

In the following work analyses have been made on material obtained from autopsies at the Winnipeg General Hospital.

Case 1. was a child, male, aged about one year (No. 463.). The autopsy was performed some sixteen hours after death, and the samples placed immediatly in glass vessels after weighing into the oven, to dry. The cause of death was obscure but was probably due to some nutritional disturbance with a secondary Bronch-pneumonia.

Case 2. was a man aged seventy six who died from Cardio-vascular degeneration (No.469). The autopsy was performed seventeen hours after death. The body appeared to be well nourished and well developed. There was very marked oedema of both lower limbs, scrotum and penis and all its tissues were quite "wet". The abdominal cavitycontained about 800 cc. of a turbid fluid. The pericardial cavity contained about 25 cc. of a blood tinged fluid. The right pleural cavity contained a little fluid but the left one contained over 800 cc. of a turbid blood tinged fluid.

The results from Case 1. are given in Table 9, along with the pathologists' remarks. The results from Case 2. are given similarily in Table 10. In Case 1. there was not sufficient material to permit of waterextractins being made, but this form of analysis was also done in Case 2.

Table 9.

Case 1. Results in mgm.Cl. per 100 gms. dried or fresh tissue

Tissue	Dry tissue	Fresh tissue	Pathologist's Remarks
Brain	605	113	Markedly congested otherwise normal.
Cartilage (cost 1)	713	197	
Duod'l Mucosa	1,249	175	
Heart	915	186	
Kidney	1,072	239	Uric acid deposits in collecting tubules. Gross appearance normal.
Lungs	921	177	Both partly consolidated and indiceed Bronch-pneumonia.
Spleen	787	184	
Pancreas	954	207	
Thyroid	893	219	

Table 10.

Case 2. Results in mgm.Cl.per 100 gms.dried or fresh tissue.

Tissue	Dried tissue	Fresh Tissue	Water extract (dry)
Cartilage	349 mgm.	135	
Heart	698	166	708
Kidney(left)	1,426	483	1,476
Liver	871	186	864
Lung (left)	1,509	255	1,503
Lung (right)	1,622	229	
Muscle (Pectoralis Major)	780	162	781
Pancreas	602	172	
Spinal Cord	630	155	
Spleen	1,014	204	1,019
Testes	2,258	270	2,240
Thyroid	1,138	216	

Pathologist's report;-

Heart: enormously enlarged--much fibrosus and replacement of muscle fibres.

Kidney: enlarged vessels--cortex and medulla scarcely differentiated--hyaline thickening of intertubular tissue.

Liver: Normal weight--extreme fatty degeneration and venous congestion.

Lung: (left) lower part completely collapsed, very congested and oedematous.

Lung: (right) twice normal weight--dark red color--cut surface poured a bloody frothy fluid--no pneumonic areas.

Spleen enlarged--diffuse perisplenitis.

Pancreas: macroscopically normal.

Note:;-

There was considerable free fluid with most of the above tissues. The cartilage was taken from the right lower costal cartilages. Due to the author's illness the above material was left in the oven for some forty-two days, at 103°. However no change of weight was noted for this period and it was concluded that the extra drying caused no material change. In spite of this long drying period the pancreas contained a large amount of fatty or oily material. I mixed this evenly with every sample examined.

Referring to Case 1. (Table 9.) it will be noticed that the results tend to be quite high, with the exception ~~which has~~ of brain and liver, which have values which one would expect in a normal individual. The high value for the

duodenal mucosa is particularily striking. This latter point may have some connection with the pathologists's suspicion of an alimentary disturbance as a cause of death. Also the fact that the subject was extremely young (one year) may account for the somewhat higher results obtained, than expected.

Referring to Case 2. (Table 10.) the most noticeable results are the extremely high values for kidney, liver, lungs, muscle and testes. These results are undoubtedly explicable by the extremely oedematous condition of the subject, or rather, that they were probably due to the same causes that caused the oedema.

These results are obviously of little use in establishing normal values. However it is interesting to note that in those tissues of which water extractions were made, no organically combined halogen was demonstrated. That is one might conclude that the chloride retention was of a physical rather than of a chemical nature. The only result to give higher values than whole blood was that for kidney. It is entirely likley that this was due to urine retention and subsequent reabsorption in the kidney tubules.

In Table 11. I have listed results by Magnus-Levy (1), Damiens (6), and St. Rusnyak & Kellner (7). The latter's method consisted briefly of digestion with Nitric Acid and Potassium Permanganate and decolorization with glucose followed by the usuall silver method of estimation. Method is probably reliable and his markedly low results are most likely due to pathological causes.

Table 11.

Results of other authors expressed in terms of fresh tissue.

Tissue	Magnus-Levy	Damiens 1.	Damiens 2.	Damiens 3.	St. Rusnyak & Kellner 1.	St. Rusnyak & Kellner 2.
Brain	130.5					
Cartilage	51					
Heart	124					
Lungs	260	240	244	230		
Liver	96	165			47	70
Kidney	208	170	207		90	108
Intestine	61					
Muscle	61				61	94
Pancreas	161					
Salivary gld.	133					
Skin					86	114
Spleen	161				74	93
Testes	226					
Thyroid	169					
Tunica alb.	332					
Total Body	123					
Child (at birth)	188 178					

Note: Damiens 1. refers to a suicide victim.
2. refers to a victim of motor gas intoxication
33 refers to a victim of accidental death.

St. Rusnyak & Kellner
1. Man, died of purulent meningitis.
2. Man, died of pulmonary tubercolosus.

Magnus-Levy's estimations were on tissues from a suicide.

Magnus-Levy's results are somewhat lower than mine with the exception of lungs and testes which are of thesame order. Possibly due to his subject being more or less normal. Damiens' results agree as far as they go and bear out the high values obtained for lung tissue.

The two cases I exmained were too markedly pathological to be of much assistance to establish normal values but it may be assumed that the halogens, particularily chlrine, are normal constituents of all the tissues of the body and possibly are in similar concentrations as in other mammals

PART FIVE .

Conclusions & Summary.

I think one might conclude from the above, that the halogens, though cheifly chlorine, are normal constituents of all mammalian tissues, and that they are in similar proportions in the various species. This is probably true for the majority of the animal kingdom, as the majority of the body fluids studied have been found to contain chlorine.

The greatest difficulty in a study such sa this is in the elimination of blood from the various tissues. As blood is markedly higher in chlorine concentration, than any of the tissues exmained with the possible exception of the kidney of Case 2., it is readily seen that the amount of blood left in them, on dissection, would materially affect their indicated halogen content. This would apply particularily to such organs as the lungs which contain a relatively large amount of residual blood on dissection.

It seems almost impossible to get blood free material or even material with a constant amount of blood. Thus, a slight difference in the distention or contraction of the peripheral arterioles and capillaries would cause a marked difference in the end result. The use of Adrenalin to overcome this last objection might be suggested but would be objected to in that the results would not be normal. In fact, one is almost justified in saying thatthe tissues dilute their contained blood and thus when one estimates the halogen content of a tissue, one is merely measuring the amount of its residual blood. However this is not entirely so as the tissues undoubtedly do contain chlorine as shown in the case of bone

and cartilage which contain very little blood but a great deal of chlorine; i.e. relatively. Similarily such tissues as the gastric mucosa undoubtedly contain more chlorine than do others, and this is not in proportion to its blood supply. One might say that although the tissues undoubtedly all contain chlorine and possible other halogens, it is almost impossible to establish their normal values until a method is evolved to determine the error caused by their residual blood.

It seems conclusive from the above work that none of the halogens are combined organically in normal or pathological tissues, with the exception of iodine in the thyroid gland. In cases of halogen retention, such as occurs in oedema, it is probable, as Snapper (8) points out, that it is due to a modified permeability of the cells. Thus as Fischer (9) shows halogen retention does not lead to oedema, but the same causes which lead to oedema, lead to halogen retention. He considers that acidosis, which leads to increased hydration capacity also leads to halogen retention.

The fact that I was unable to demonstrate the presence of organically combined chlorine in the gastric mucosa is significant.

In Table 12, I have summarized the mean halogen content of the animals exmained along with Magnus-Levy's figures for man, in milligrams of chlorine per hundred grams of fresh tissue.

Table 12.

SUMMARY.

All results expressed in mgm.Cl. per 100gm. of FRESH tissue.

Tissue	Rat	Dog(6)	Child(1)	Man(2)	Magnus-Levy
Adrenals		124			
Bone	125	112			
Brain	113	148	113		131
Cartilage		180	197	135	51
Duodenal Mucosa	84	124	175		(gut)61
Heart	111	118	186	166	124
Kidney	178	251	239	483	208
Liver	132	136	134	186	96
Lungs	196	235	177	255(L) 229(R)	260
Lymph Glds	156				
Muscle(vol.)	60	67		162	61
Ovaries	290	190			
Pancreas		159	207	172	161
Spinal Cord		118		155	
Spleen	134	171	184	204	161
Salivary glands	125	152			133
Stomach mucosa	136	160			
Tendon		261			
Thyroid		161	219	216	169
Testes	222	187		270	(TUN.A.)332
Uterus		201			

Note: For Magnus-Levy's figures for testes 332 mgm. refers to the tunica albuginea.

In considering these results it might be remembered that each drop in the titration accounted for a difference of approximately two (2) milligrams of chlorine per hundred grams of fresh material. This error was approximately the same for both micro and macro proceedures.

In connections with this study it is interesting to note the results of the investigations of Rosemann (10) and others. He found that the highest total halide content of a human foetus was 270 mgm per hundred grams fresh material and that this value decreased with the development of the foetus until it reached a value of 120 mgm in adult man. This may be one reason for the relatively high chlorine content found in the child (case 1.) exmained, above.

The author is greatly indebted to Professor A. T. Cameron, under whom the above work was carried out, and to Dr. F. D. White for their kind assistance; also to Professor Wm. Boyd, of the Pathology department, for assistance in obtaining autopsy material.

REFERENCES.

(1) Magnus-Levy, Adolf. 1910 Biochem. Zeitsch 24. pg. 363-380
(2) Van Slyke, D. D. 1923. J. Biol. Chem Lviii. pg. 523-529
(3) Lawrence & Harris, J.V.&J.A. J.Am.Chem.Soc. 46. pg.1471-7
(4) Cameron A. T. 1927, Can. Med. J. XVII, 670,675.
(5) Kendall E. C. 1920 J. Biol. Chem. XLIII, No.1 149.
(6) Damiens, A. 1920. Compt.rend. 171, pg.930-933

1920 Bull.des Sc.Pharm. No.12 &1921 no. 1,2,4.
(7) St. Rusnyak & Kellner,D. 1922 Biochem.Z. 133 pg.523-29
(8) Snapper, J. Deut.Arch.klin.med. 111, pg.441-62.
(9) Fischer M. H. 1915 J. AM.MED.ASSOC. 64, pg. 325-6.
(10) Rosemann,Rudolf. 1911 Pflugers Archiv. 142 pg.441-82
(11) Bell,R.D. & Doisy E.A. 1921 J.Biol.Chem. 451, pg 427-35.
(12) Goto,Kiko 1922 Tohoku J.Exptl. Med. 3, pg. 1925-205
(13) Le Calve! J. Compt.rend.soc.biol. 73, 74.
(14) Leva, J. 1915 Berlin Z.klin.med. 82, pg. 363-380
(15) Weinberg, Benjamin. Bertr.Geburtsh.Gynaekol. 19, pg.222-35.

A THESIS

Part 2.

entitled

THE BIOCHEMICAL METHODS INTRODUCED BY BOURQUELOT AND HIS SCHOOL, AND THEIR APPLICATION.

presented to

THE DEPARTMENT OF BIOCHEMISTRY
of
THE UNIVERSITY OF MANITOBA

by

C. H. A. WALTON

as partial requirement for the degree of

Master of Science

April 1928

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- b.) Scope of present work

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Detailed account of the various methods used in the attempt to isolate the fluorescent glucosides from the stems of Symphoricarpus occidentalis Hook (Wolfberry), and from the roots of Diervilla diervilla (Bush Honeysuckle).

Part Three

Conclusions and Summary.
References.

PART ONE.

Introduction.

McCullagh (1) while working on an extract of Wolfberry Stems, (Symphoricarpus occidentalis, Hook), examining it by Bourquelot's biochemical method, discovered that after clarfication with neutral lead acetate, and making alkaline with dilute Ammonia, a very marked deep blue fluorescence was obtained, but that this fluorescence did not appear in the flask treated with emulsin. He concluded that the fluorescence was due to a Beta - gluoside, hydrolysable by Emulsin (unpublished).

It occurred to him that this property of fluorescence would serve as an indicator and thus aid materially in the isolation of the glucoside. He found, however, that it was extremely soluble and impossible to obtain pure, or at least impossible to obtain a crystalline product.

It was decided then that I should carry on from the point he left and attempt to isolate the glucoside from Wolfberry Stems, in the hope that a method might be developed which would be suitable for the isolation of other glucosides, whose presence was indicated in McCullagh's previous work (1), and in related species examined by the various French Workers. Accordingly I gathered several kilograms of Wolfberry Stem, in September 1927, and prepared Bourquelot (2) extracts as follows:-

As soon as possible, after collection theleaves were stripoff, and the stems chopped into short lengths andthrown into boiling 85% ethyl alcohol. This mixture was boiled under a reflux condenser for thirty minutes, and the resulting green alcoholic extract decanted off, and the residual stems again extracted with an equal quantity of 85% ethyl alcohol. The two extracts were com-

bined and reduced to small bulk under reduced pressure, and the resulting aqueous extract made up with thymol water to a volume such that I cubic centimeter was equivalent to 1 gram of fresh stem.

Several glucosides are known which give blue or green fluorescence in alkaline or acid extracts, which appear to have been fairly readily isolated in the pure crystalline state. All these fluorescent glucosides are Hydroxy-coumarin derivatives.

Two of the commonest fluorescent glucosides, that is, those fluorescent in alkaline media, are Aesculin and Fraxin both of which are readily crystallizable from hot water or dilute alcohol, as they are somewhat insoluble. The great feature of the fluorescent glucoside which we have investigated is that it seems to be extremely soluble in the commoner solvents and when precipitated by ether or acetone it comes down in an amorphous form. In other words it seems to be either completely insoluble or very readily soluble in the cold. In the following work I attempted to prepare a pure preparation by various methods attempted by McCullagh who thought he had attained a relatively pure preparation and by methods which I devised later.

PART TWO.

Preparation 1.

Filtered 500 cc. of the extract described above, and clarified by addition of excess neutral lead acetate, filtered, and subsequently removed the excess lead with Hydrogen Sulphide. Excess H_2S was removed by passing a rapid current of air through the solution. To this solution, which had now a volume of nearly 900 cc's. I added an excess of Basic Lead Acetate and filtered off

the relatively small yellow preciptate obtained. Took this precipitate up in a minimal quantity of dilute acetic acid and removed lead in the usual way, and took the solution down to dryness at 50°C. in vacuo. I extracted the small quantity of dry residue with pure methyl alcohol, and precipitated it from the alcohol by excess of acetone. The yield obtained was very small and hygroscopic, being almost impossible to dry.

I took the filtrate from the Basic Lead (above) and added an excess of Ammonia which threw down quite a heavy precipitate of lead and apparently took with it all the remaining fluorescent material. I treated this precipitate as above and obtained finally a much larger yield of an amorphous buff colored material which dried easily and was not hygroscopic. I considered that this was probably a highly impure preparation. In this preparation, I noticed the following points of interest. Firstly the precipitate obtained with basic lead was disappointingly small. Secondly that the preparations were extremely fluorescent in methyl alcohol and imparted a red color to the solution. Thirdly that they were precipitated with difficulty from the alcohol by acetone, and required large quantities of acetone. The acetone precipitates were always extremely gummy and hard to dry. Fourthly and the most important, I found that my preparations were soluble in excess of basic lead solutions. They shared this property with a crystalline sample of Aesculin (B. D. H.). Obviously in future preparations I must be careful not to add an excess of basic lead acetate solution.

My chief reason for doubting the purity of either of the above preparations was that they were not markedly fluorescent, even in concentrated solutions. I will show later that fluorescence is <u>not</u> a <u>quantitative</u> property.

I decided to try another type of preparation, as it was thought that the acetic acid, used to take up the basic lead precipitate was hydrolyzing the glucoside. I decided to adopt a method previously worked out by McCullagh.

Preparation 2.

I clarified a litre of the Wolfberry extract, as in (1) and treated it very carefully with a concentrated solution of basic lead acetate (prepared as described below), filtering after each addition, to note whether any further precipitate was forming. I dissolved the precipitate thus obtained in a minimal quantity of dilute acetic acid, giving a yellow solution. Removed the lead in the usual manner, and divided the resulting solution into two parts "A" & "B".

"A". Added potassium hydroxide solution to exact neutrality and reduced to dryness at 40 ^{o}C., under reduced pressure. Attempted to extract the resulting potassium acetate in the residue with boiling absolute alcohol. This proved to be an abortive proceedure as the glucoside also dissolved in the boiling absolute alcohol, as evidenced by its very marked fluorescence. Obviously any idea of neutralizing the acetic acid in this manner is useless particularily in view of the fact that a large quantity of inorganic salt is introduced which is difficult to get rid of later. McCullagh was led into this proceedure by an observation he had made on his supposedly pure preparation indicating that the glucoside under investigation was insoluble in absolute alcohol, even in the hot. Obviously he was wrong, and this indicates that his preparation was probably very highly impure.

"B" Took other half of the above lead free solution down to dryness at 40 ^{o}C under reduced pressure, and extracted several

times with hot methyl alcohol and filtered. A slight insoluble residue was left. On taking the methyl alcohol extract down to dryness a yield of 295 milligrams was obtained. The insoluble residue was soluble in water and was fluorescent. At the time, I thought that this might indicate an insoluble split product, but I now think that it was most likely impurity containing a trace of adherent glucoside. I found later that infinitely small traces of the glucoside gave remarably brilliant fluorescence, almost it seemed in an inverse proportion to the concentration of glucoside. The residue wastoo small to work on any further and was discarded.

Although this last preparation "B" was probably fairly pure I decided that another method must be devised to obtain larger and purer yields, so that the glucoside might, at least be identified.

Preparation 3.

In the previous preparations, aqueous solutions invariably reduced Benedict's(5) quantitative solution, so it was thought that the chief impurity was some form of hexose, probably glucose and that these sugars possibly prevented the product from crystallizing. Accordingly it was thought that if an extract of the Wolfberry stem was treated with yeast the sugars would be removed by fermentation, and thus facilitate the preparation of the pure product. Obviously this yeast must be free from any Beta-Glucosidase.

McCullagh found, last year, that a brewer's top yeast from Shea's Winnipeg Brewery met this specification. I obtained some of this and filtered it dry, and preserved the dry yeast in an ice chest. I tested it as follows:- yeast was added to four vessels containing 1% Amygdalin (B.D.H.), distilled water, - 1% Amygdalin plus invertase, and distilled water plus invertase, and the four

four solutions incubated at 37°C. for three days. All four were tested qualitatively by Benedict's reagent, and a negative result obtained in each case, showing that the yeast was free from any Beta-Glucosidase or sugars. This result was checked by the fact that no characteristic odor of Prussic Acid was noted which would have been present had any of the Amygdalin been hydrolysed.

To 500 cc. of the plant extract (Wolfberry stem) I added a quantity of yeast and incubated at 37°C. for six days. At the end of six days, controls of sucrose and yeast gave negative Benedict reactions indicating complete fermentation.

The mixture was now treated with neutral lead acetate solution until no further precipitate was obtained, filtered, and the lead and excess H2S removed from the filtrate in the usual way. The filtrate was then taken down to dryness under reduced pressure, at 40°C, and the dried extract extracted with hot methyl alcohol several times. A dirty grey residue remained which was soluble in water and fluorescent. On cooling some supposed glucoside settled out in an amorphous cloud which redissolved on addition of excess methyl alcohol. The clear methyl alcohol extract was a bright red in color with a particularily deep blue fluorescence. This was taken down to dryness at 40°C. under reduced pressure, and yielded a light buff colored material of similar appearance to the former preparations. Yield 3.350 grams. This material was amorphous, and of a light buff color, soluble in cold and hot water, methyl alcohol, and <u>hot</u> absolute ethyl alcohol. It is precipitated from the alcohols in an amorphous mass by ether and acetone, several volumes of which are required.

The fact that it was soluble in hot absolute alcohol, leaving only a small residue, suggested that this might be a use-

ful means of purifying it. Accordingly, I took 3 grams of the preparation and extracted it repeatedly with small quantities of hot absolute alcohol, and filtered through a steam heated filter. On cooling, a very gummy precipitate settled out which gave no signs of a crystalline structure. The material was so gummy that I took it up in hot methyl alcohol and reduced it to dryness obtaining 1.05 grams of a reddish brown material. Incidentally, the methyl alcoholic solution was red and intensly fluorescent. I also treated the residue from the hot absolute in a similar manner obtaining a little over a gram of material. Obviously this proceedure is too costly in material, and of no apparent use as a purifying method. The absolute alcohol preparation remained very gummy after the methyl alcohol treatment; even taking it up in water and subsequently drying did not improve it. It appeared that the absolute alcohol had brought about some change, perhaps in the nature of a dehydration. These preparations were used for hydrolysis tests described below.

<u>Preparation 4.</u>

I repeated the above proceedure (3) on 500 cc. of extract, incubating for seven days, at the end of which a sample reduced Benedict's reagent, accordingly I incubated for a <u>further</u> seven day period. (The yeast was tested for Beta-glucosidase as above). The mixture was then clarified in the usual manner with neutral lead acetate. Reduced the solution to dryness and took residue thus obtained up in a minimal quantity of distilledwater (150 cc.).

Thinking that the fault in the first preparation (above)

lay with the basic lead acetate (prepared as described below) I decided to use a solution of basic lead acetate prepared as advocated by Bertrand and Thomas (4).

To the aqueous extract I added, cautiously, basic lead, prepared by this latter method, filtering after each addition, until no further precipitate was obtained. Took the lead precipitate up in a minimal quantity of dilute acetic acid and removed the lead in the usual manner. This was taken down to dryness at 40°C, under reduced pressure, and extracted with hot methyl alcohol, leaving a dark colored impurity, and the alcoholic solution again reduced to dryness. Yield about 600 milligrams. This preparation was of a similar appearance to the former ones; it was soluble in water methyl and 95% ethyl alcohols, and insoluble in ether and acetone. It appeared to be insoluble in absolute alcohol. All attempts at recrystallization were unsuccessful, and I considered that this preparation undoubtedly contained a great part of the glucoside but was relatively impure.

I hydrolyzed a little of it in aqueous solution, using a drop of concentrated hydrochloric acid. A fruity odor was noted, and on cooling a precipitate formed which on centrifuging off was amorphous, insoluble in cold water, soluble in aqueous alcohol, and non-fluorescent. On making alkaline with a little Ammonia the mother liquor did not give the usual fluorescence, indicating that it had been destroyed by hydrolysis. The balance of this preparation was used in hydrolysis tests, subsequently.

<u>Preparation 5.</u>

I had a supply of Wolfberry stems collected on January

17th, 1928, and prepared a Bourquelot extract as in other cases. On clarifying with neutral lead the solution was quite fluorescent indicating that the glucoside remained in the stems, at least in part, during the winter season. However, no precipitate was obtained with basic lead acetate (5) and it occurred to me that the glucoside was not precipitated by it. I hydrolyzed a little in a test tube with a little Sulphuric Acid, neutralized with Baryta, and added a little basic lead acetate, and obtained a heavy precipitate indicating that perhaps the split product only was precipitated by basic lead, and that the split product did not exist in the stems in winter.

I placed the whole clarified extract in a flask connected to a reflux condenser and hydrolysed for three and a half hours with 2% Sulphuric Acid. I noticed that within a few minutes large charred pieces formed on the sides of the vessel. This was noticed in all the subsequent hydrolyses. Also a very characteristic pleasant fruity odor was given off, and an oily material was noticed to condense in the condenser, indicating the presence of a highly odoriferous steam volatile oil.

The hydrolysate was neutralized exactly with baryta, and treated with basic lead, with very poor success, and in the subsequent manipulations most of the active material was lost. I attribute my failure in this case to the fact that both the glucoside and split product are readily precipitated by basic lead acetate at the correct Ph. i.e., slightly alkaline, and only under that condition. I found, in test tube tests, that addition of a drop of ammonia very often produced an extremely heavy precipitate, when using basic lead, a precipitate vastly greater than would be due to the formation of lead hydroxide. It will

be noted that I did not take the necessary precautions, along this line, in all my preparations up to this point. Another reason for failure in this case is undoubtedly due to insufficient washing of the lead precipitates, after Hydrogen sulphide. (Note the hydrolysate was non-fluorescent).

<u>Preparation 6.</u>

Some concentrated extracts prepared by a "yeast procedure" by McCullagh were reduced to a thick syrup to remove the alcohol, in which they were in solution, and the syrup taken up in a little water. Both the alcoholic solution and the aqueous one were red in color and exhibited a deep blue fluorescence. The whole was hydrolyzed with sufficient concentrated Sulphuric Acid to turn Congo Red paper blue, for three and a half hours. The charring noted in preparation 5 was also noted here. On cooling a small precipitate separated which was brown in color, and soluble in 95% alcohol. I considered that this was probably impurity from the yeast, but as I will show later I may have been mistaken in this surmise.

On neutralizing a small quantity with Ammonia a brilliant blue fluorescence was developed which seemed to contradict all other observations on hydrolysis of this particular glucoside. I took a third of this mixture and neutralized it carefully to litmus with Ammonia and extracted several times with small quantities of ether until the ether was no longer colored yellow; the aqueous phase remained fluorescent. The yellow ether extract was taken down to dryness, giving a small yellowish white residue. A little of it in water was non-fluorescent, even with ammonia. I took the dried ether residue in 95% alcohol in the hot, which became cloudy on cooling. Addition of water didnot remove the

cloudiness, but addition of acetone immediately cleared the alcoholic solution. Thus I concluded that it was insoluble in water but soluble in aqueous alcohol, and acetone, ether, etc. On addition of a little water to the alcohol acetone mixture, a green fluorescence developed. The alcoholic solution gave a green catechol reaction with fresh aqueous Ferric chloride. It seemed that this might represent either the split product or a derivative of it. However, there was very little to work on, and all attempts at recrystallization were useless.

I took the 1/3 of the solution, extracted by ether (above) and reduced it to dryness under reduced pressure at 40°C, and extracted it with ether in a Soxhlet apparatus for over 24 hours. On evaporating off the ether it was seen that nothing had been extracted. Although it was reasonable to expect that a coumarin derivative, such as this might be soluble in ether this seemed to indicate that it was not. In the light of later work, however, it is possible that this failure was due to incomplete hydrolysis, and that any little split product formed had been extracted previously by the ether, from the aqueous mixture,. On taking this material up in a little water it was reddish in color and highly fluorescent.

To this aqueous solution I added 5 cc. of basic lead acetate solution, producing an amazingly heavy precipitate. Took this up in dilute acetic acid, forming a thick cream, so as not to use too much acetic, and removed lead in the usual way, and took down to dryness in the usual manner. The resulting residue was very gummy and extremely difficult to dry. In aqueous solution it readily reduced 8% potassium permanaganate. I attempted to oxidize a little with nitric acid, and nothing was precip-

itated with ether. Ether precipitates it from alcoholic solution, but unfortunately as a dirty amorphous material.

I boiled half of the product with dilute nitric acid for half an hour, and extracted with ether. The ether extract was found tocontain nothing, as was the aqueous phase; this seemed to indicate a more or less complete destruction by the nitric acid. Similar treatment with oxidizing agents such as hydrogen peroxide, etc., seemed to indicate that the product was readily oxidized but that in the process of oxidation the phenyl ring was ruptured, instead of the formation of hydroxy benzoic acids as one might expect from the oxidation of a coumarin derivative.

I took the balance of the hydrolyzed material (2/3), and distilled over a free flame. Unfortunately breakage of the distilling flask lost most of the material, but the aqueous distillate contained the highly odoriferous substance noted in all the hydrolyses attempted. I extracted this distillate with ether and on evaporating the ether obtained a slight amount of solid, which was non-crystalline, and non-fluorescent. It was of too small a quantity to work on but undoubtedly represented the steam volatile odoriferous substance. It is possible that it is of the nature of an essential oil, as subsequent work indicates that neither the split product nor the glucoside is steam volatile.

Preparation 7.

In 1911, Charaux (6) found a large amount (3%) of Fraxin in an extract of Diervilla lutea roots. Fraxin is the glucoside of methoxy tri-hydroxy coumarin, which probably is closely related to the glucoside which I am trying to isolate. Accordingly I attempted to isolate it using a method allied to his.

Two hundred cc's. of a Wolfberry extract, collected in September 1926, by McCullagh, were filtered and extracted several times with large quantities of ether, in an endeavour to precipitate a saponin like material which Charaux considers holds the otherwise insoluble Fraxin in solution. The ether extracted a yellow material, but it was not worked up. The aqueous extract was then evaporated down to about 50 cc. bulk, at 60°C under reduced pressure, in the hope that the glucoside might crystallize out. However, before this reduction in volume the solution was filtered; it filtered very slowly leaving a very gummy residue on the filter paper, indicating that the ether had undoubtedly removed something analagous to the so-called saponin found by Charaux. When reduced to small bulk the solution was a dark red brown in color, whereas, before the ether extraction it was a light brown in color.

After reduction to fifty cc. nothing crystallized out, so I again extracted with ether as before and filtered; filtration was again very slow. Further reduction in volume did not bring about the desired crystallization.

It was now suggested that the ether had not removed all of the saponin, and that this might be done by addition of three volumes of 95% ethyl alcohol and three volumes of ether. This treatment brought down a fairly heavy brown precipitate which was filtered off. The residue was extracted with a little warm 95% alcohol, and proved to be fluorescent, indicating that some of the glucoside had been carried down by the precipitate, i.e., adsorbed. The filtrate was again reduced to a small volum, but as before nothing separated out.

I then took it down to dryness and extracted with hot methyl alcohol which left a slight dirty looking precipitate,

which was discarded. This methyl alcohol extract was red in color, and very brilliantly fluorescent.

An equal volume of ether was added, producing an extremely dense white precipitate which was filtered off. The filtrate being no longer fluorescent I discarded it, considering that the ether had precipitated all fluorescent glucoside. I took the ether precipitate up in hot 95% ethyl alcohol, leaving an insoluble gummy material, which was discarded. Part of this alcoholic solution "A" was treated as follows; "B" the other was treated as described below "A".

"A". I noticed that addition of one or two volumes of acetone intesified the fluorescence, and of course no separation of the liquid phases occurred. On addition of a similar quantity of ether, the aqueous phase was of course thrown down, and the ether-acetone phase was noticed to contain a very brilliant fluorescence; i.e., the glucoside had been partially extracted by the mixture; that some remained in the aqueous mixture was shown by its continued fluorescent properties. Of course this phase was considerably concentrated by the treatment, and it assumed a dark opaque appearance, and contained brown gummy specks in suspension. That the glucoside should be extracted by an ether-acetone-water mixture in this fashion, when the glucoside was obviously insoluble in either either or acetone, suggested that this might constitute a good method for the extraction of the glucoside. On reducing to small bulk the ether-acetone extract became cloudy. The precipitate was not crystalline, and was small in bulk. On centrifuging it appeared to be very slightly soluble in water, soluble in 95% ethyl, and pure methyl alcohols, in which it was fluorescent, but insoluble in absolute. The

yield was too small to work on so I determined to work up "B" which contained the bulk of the material.

The aqueous solution was about thirty cc.'s in bulk, and was treated as follows:- Two volumes of acetone were added to it, in a separatory funnel, and well shaken. Two and a half volumes of ether were then added, and the two phases separated in the usual manner. This extraction process was repeated several times, and the volume of the aqueous solution was made up to thirty cc's. from time to time, with distilled water. Took this ether-acetone extract down to dryness, yielding a buff colored amorphous material, of about 660 milligrams. This substance appeared to be fairly soluble in water, soluble in 95% ethyl, and pure methyl alcohol, from which it was readily precipitated with ether. It was, however, apparently insoluble in absolute alcohol.

Its aqueous solution gave a precipitate with neutral lead acetate indicating that although a purification had been undoubtedly affected clarification initially with neutral lead acetate was desirable. For example it was found that an aqueous extract of Quercitin (E.K.Co.) was readily extracted by this proceedure, and as all such flavones are readily precipitated by neutral lead this treatment appears to be doubly necessary. Although the ether-acetone extraction proceedure unquestionably affects a considerable purification it does not eliminate any possible contamination with glucose. Glucose is readily extracted from its aqueous solution by this proceedure.

These relatively impure preparations were used in a series of emulsin hydrolyses to be described later.

Preparation 8.

Throughout the remainder of this thesis I will refer to the proceedure adopted in the last preparation as the ether-acetone extraction method.

An extract (500 cc.) of roots of Diervilla diervilla, the Bush Honeysuckle, collected by McCullagh in September 1926 was examined at this point. It was considered that it might yield interesting results in that it was very closely allied to Diervilla lutea in the roots of which Charaux (6) had found such large quantities of Fraxin.

The extract was clarified with neutral lead in the usual manner, and reduced in volume to fifty cc's., and extracted by the ether-acetone proceedure, outlined above. (Only half of this extract was used) A yellow amorphous powder weighing about 800 milligrams was obtained.

Properties: soluble in water, 95% and absolute ethyl alcohol, and in methyl alcohol. In alcoholic solution it gave a brilliant green-blue fluorescence definitely different in appearance from that observed with the Wolfberry. All attempts at recrystallization of this product were unsuccessful, so that its purity was open to question. One attempt at purification consisted in solution in absolute alcohol, and precipitation with about seven volumes of ether. It was thrown out in a brilliant yellow floculent precipitate which was not crystallin, but slightly more difficult to dissolve in absolute alcohol. The preparation was definitely insoluble in ether, acetone, or ethyl acetate. However, it was soluble in an acetone water mixture. It is soluble in glacial acetic acid from which it is precipitated by ethyl acetate in an amorphous mass.

This preparation gives a very deep green with a little fresh aqueous Ferric Chloride, which changes to a deep red on addition of one or two drops of 0.4% caustic soda. This reaction is typical of all coumarin glucosides, and derivatives and indicates a cathechol nucleus (7). It readily reduces 8% Potassium permanaganate in the cold, in the presence of a little dilute sulphuric acid, indicating the possible presence of a double bond, or at least that it is very readily oxidized.

This preparation and the unextracted portion were subsequently used in hydrolysis tests to be reported below.

Preparation 9.

I concluded from the above work that the best chance of obtaining a product approaching anything like purity, lay in using this acetone-ether extraction method after clarification with neutral lead acetate. Accordingly, I filtered the balance of the extract which I had obtained in September 1927, ~~of Wolfberry stem~~ namely, 500 cc., and clarified with neutral lead acetate in the usual manner. I then reduced the whole solution down to about 100 cc. bulk and made the ether-acetone extract as described, previously. On reducing to dryness I obtained an almost colorless amorphous material of about 2 grams weight. From the properties outlined below it will be seen that this preparation probably was almost pure glucoside.

Properties: Soluble in cold water, methyl alcohol, 95% and absolute ethyl alcohols. Insoluble in ether, acetone, and ethyl acetate. It was soluble also in glacial acetic acid. It requires hot absolute to dissolve it but it does not come out on cooling. Its aqueous solution is very fluorescent on the addition of a drop of 14% ammonia. In alcoholic solution it is ex-

tremely brilliantly fluorescent even in neutral or slightly acid solutions. All attempts at recrystallization from water, or alcohols etc., were unsuccessful, so that this criterion of purity was lacking.

With fresh ferric chloride solution it gives an intense green color which turns to a deep red on addition of a few drops of 0.4% NaOH. This is the typical reaction given by all the coumarin glucosides and of course indicates the presence of a catechol nucleus. On heating it turns red brown with ferric chloride, and a red brown precipitate is thrown down on cooling, indicating a possible oxidation. With potassium permanagante it is reduced readily in presence of dilute sulphuric acid.

On drying the ether-acetone extract it tended to become gummy, and it was necessary to dry with four washings of absolute alcohol, and two of ether, for a period of nearly four hours. The ultimate product was not deliquescent at all. For its weight the preparation was surprisingly bulky.

The aqueous solution of this preparation is yellow in color, and in concentrations of over 1% is cloudy; even centrifuging does not remove this cloudiness. I attempted to take polarimetric readings on aqueous solutions made alkaline with a little ammonia, had found that the greatest concentration through which a reading could be made was 0.5%. Readings of such a solution, using a one decimeter tube, were of the order of minus 0.26° which would indicate that is a laevo-rotatory substance having a specific rotation of -52°. It was found that alcoholic solutions were less colored and clearer, and polarimeter readings on a 1% alcoholic solution (90% ethyl) of the preparation, indicated a specific rotation of minus 44

As the solutions investigated were so dilute these readings are necessarily somewhat inaccurate, but one can at least assume that the preparations is definitely leavo-rotatory having a specific rotation in the region of minus 45°.

I prepared ozasone crystals from the preparation (1%) both before and after hydrolysis with dilute hydrochloric acid. After boiling with the phenylhydrazine mixture for three-quarters of an hour and allowing to cool slowly for an hour, a small crop of definite glucozsasone crystals came down. These were not mixed with any other type of crystal. This indicated that glucose existed in the preparation as an impurity, or more probably that a small quantity of the glucoside was hydrolysed by the boiling. After hydrolysis, an extremely heavy crop of crystals came down on boiling about ten minutes with the phenylhydrazine mixture. This indicated that glucose had resulted in large quantities from the hydrolysis, This hydrolysis of course would involve that of any sugar such as sucrose, present as an impurity. As I will show later sucrose is not present in this preparation. As glucosozone crystals were the only type formed I think we might reasonable conclude that the sugar part of the glucoside molecule is undoubtedly glucose.

The melting point of the preparation is about 95°C. I know of no other glucoside having a melting point below 100°C, and of no coumarin having one below 140°C. In fact Fraxin has a melting point of 350°C. I conclude that this indicates the presence of an impurity, in reasonably large amount.

As I mentioned above this glucoside is possibly resulted to Fraxin, which is methoxy derivative of a trihydroxy coumarin. Accordingly I Performed two methoxy determinations by

Zeisel's method (7) and indicated the presence of one or more definite methoxy groups. The first estimation indicated that the CH_3O amounted to 6.2%, and the second determination indicated that it amounted to 7.25%. As the estimation was performed on 300 and 200 milligrams respectively the agreement is relatively close. Assuming that the glucoside consists of one glucose molecule attached to a methoxy-di-hydroxy coumarin we would have a molecular weight of about 370 of which CH_3O would be 8.3%. My results seem to indicate that the new glucoside contains one methoxy group, and that the preparation is possibly 80% pure.

I considered that the chief impurities might be sucrose and glucose. I hydrolysed a small quantity with dilute hydrochloric acid for two minutes, cooled, and adding Seliwanoff's reagent boiled for over thirty seconds. There was no indication of the presence of fructose so that one might conclude that sucrose is not an impurity. Ordinarily one might test for glucose by a copper reduction test such as Fehlings or Benedict's. However, this is impossible with such substances as hydroxy-coumarins. It is well known that substances with adjacent hydroxyl groups on a phenyl ring, like Catechol and Pyrogallol in alkaline solution, readily reduce copper, gold and silver salts etc. I tried a Benedict, Fehling, and Bertrand test on a dilute solution of pure crystalline Aesculin (B.D.H.). In all cases a very marked reduction occurred. As Aesculin has two adjacent hydroxyl groups, and is a coumarin derivative I considered that such reduction tests were useless to indicate the presence of absence of glucose from my preparation. How-

ever, it was thought that the performance of a quantitative Benedict (8) estimation, before and after hydrolysis, might indicate whether one or more glucose radicles occurred in the new glucoside molecule. However, I found that this was impossible of execution in that the oxidized products of the glucoside colored the solution either dark brown or black thus definitely obscuring the endpoint. This darkening of the solution on oxidation is, of course common in such substances as pyrogallol. Obviously Benedict's method is useless in any estimations on glucosides, such as are necessitated in Bourquelot investigations. Even in dilute solutions such as are used in Bourquelot estimations a masking of the end point would occur, where ever hydroxyphenyl compounds occurred in the solutions examined. Thus McCullagh's estimations (1) are open to criticism in this particular.

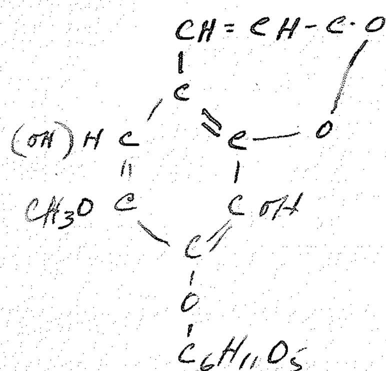
I considered that the only way in which the reduction of Cupric salts could be used to estimate sugar in my preparation was to use some method in which the Cuprous oxide is removed from the solution and examined separately. Such a method is that of Bertrand and Thomas (9). They estimate the cuprous oxide formed by filtering it off, treating it with an excess of Ferric Sulphate, and titrating the amount of Ferrous Sulphate formed, by means of Potassium permanaganate. I employed this method in estimating the reduction value of my preparation, before and after hydrolysis, in terms of glucose. It is a significant point the French workers invariable use this latter method in all their Bourquelot estimations. Undoubtedly it is the only suitable method.

Results of Bertrand estimations on this preparation.

40 mg. of preparation before hydrolysis gave a reduction value of 5 mg. glucose.

50 mg. of preparation after hydrolysis reduction of 30 mg. glucose.

Thus the unhydrolyzed material gave results indicating 12.5% glucose and the hydrolyzed material - 60% glucose, i. e., one might conclude that glucose liberated by hydrolysis amounted to 47.5% assuming that my preparation has a formula, corresponding to this one would expect a glucose content of 48.6% for a di-hydroxy and 47.2% for a tri-hydroxy coumarin glucoside.



$C_{16}H_{18}O_{10}$ → molecular weight of 370 of which glucose, 180, is 48.6%. Supposing another "OH" group in the 5 position molecular weight becomes 386, with glucose 47.2%.

I consider that these results indicate a reasonable purity of my preparation, in fact a much greater purity than indicated by the methoxy determinations. As I have mentioned above the hydroxy phenyl compounds are reducing so one might conclude that the 12% glucose indicated in the estimation before hydrolysis, indicated the reduction value of the hydroxy parts of the phenyl ring. In fact the conclusion is almost warranted that glucose is not an impurity in this preparation.

To further settle the structure of the supposedly new glucoside one would have to perform a large number of analyses. I think that the Bertrand estimation definitely shows that the

moleculecontains only one glucose radicle. As I have been unable to obtain an obviously pure preparation it would seem useless to perform any other tests on it. The idea of preparing a crystalline "Split-product" suggests itself, particularily in view of the fact that most of the coumarin derivatives are relatively insoluble and hence easy to crystallize. Accordingly I propose to perform the following series of analyses on the "Split-product" when it is obtained.

1. Molecular weight determination by combustion.
2. Estimation of hydroxy groups by acetylation (10)
3. Estimation of double bonds by treating with hydrogen.
4. Further methoxy determinations.

Attempts at Hydrolysis.

Up the time of writing this report I have been unable to obtain any large quantity of the hydrolyzed product, so that a report of methods would be premature. I will simply give a brief account of my work in this particular.

In all cases hydrolyses were carried out on relatively small amounts of impure extracts and occasionally in small quantities of purer preparations. This was done in order that I might economize on.material.

Several preparations were hydrolyzed with 2% Sulphuric Acid under a reflux conclusion for 3 or 4 hours. On cooling a precipitate settled out which was non-crystalline and soluble in aqueous alcohol. One hydrolysis was attempted with 2% Sodium Hydroxide and several with Emulsin (B. D. H.). Hydrolyses were also attempted under reduced pressure and after steam distillation. To date I have obtained the following information from the above.

1. The "Split-product" appears to be colorless, crystalline (needles), and to have a melting point ofover 220° C.

It is insoluble in cold water and soluble in boiling water. It is soluble in aqueous alcohols, ether, and ethyl acetate. It appears to crystallize readily from hot water except when in the presence of an impurity which has a low melting point and is removed by steam distillation.

It was found that this "impurity" seems to hold it in solution after hydrolysis, although the precipitate after acid hydrolysis is undoubtedly mostly "Split-product". Accordingly I extracted all hydrolyzed mixtures with ethyl acetate; took this extract down to dryness and extracted it with ether. The ether extract was usually easy to crystallize from boiling water.

In the case of the Emulsin Hydrolyses the ethyl acetat formed an Emulsin which necessitated the use of acetone to liberate it, as a separate phase. The ethyl acetate was always colored yellow. On addition of a little alkalie the yellow color was greatly intensified and all of it settled out to the bottom. In other words ethyl acetate only extracted neutral or acid solutions.

At present I am attempting preparations on a larger scale using the following technique.

As the use of acid or alkalies is undesirable due to the difficulty of getting rid of them. I decided to use Emulsin. This Emulsin was a product of the British Drug Houses Limited. I tested its activity on Amygdalin and obtained signs of hydrolysis after 10 minutes incubation at 37°C., as evidenced by the strong odor of prussic acid, and strong reducing properties, neither of which were in evidence before the treatment.

An extract of the stems was clarified with neutral lead with usual manner, and the resulting solution taken down to dryness to remove the acetic acid. Took the dry residue up in a little water. It was intensely fluorescent. I added a little Emulsin and incubated for 36 hours at 37°C. The solution lost its fluorescence by this treatment. It was now boiled to precipitate the Emulsin and dissolved any "Split-product" which may have come out of solution ; it was filtered hot and residue discarded.

This solution was then extracted with mixtures of ethyl acetate and acetone. The use of acetone produced a brilliant fluorescence indicating that the hydrolysis may have been incomplete. However, I have been unable to decide yet, whether or not the "Split-product" is fluorescent.

> Note:- If so called "absolute"ethyl acetate (Merck) is used - acetone is required to "crack" the resulting Emulsin. However, if 90% ethyl acetate (Squibb's) is used its 10% alcoholic content seems to overcome this difficulty thus making the use of acetone unnecessary.

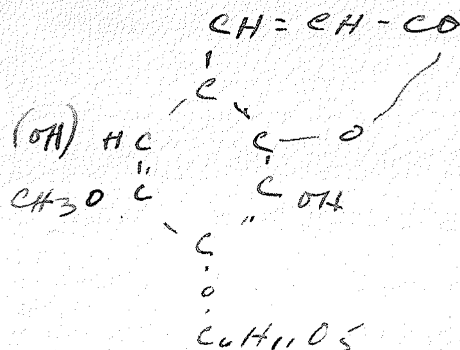
I am unable to report further progress on hydrolysis preparations. However, I might report that the "Split-product" from the roots of D. diervilla appears to be very similar to that from the Wolfberry.

PART THREE.

Conculsions and Summary.

I think I am justified in concluding that Symphoricarpus occidentalis Hook (Wolfberry) stems contain a fluorescent glucoside, hitherto not identified or isolated. It seems to be a glucose derivative of a mon-methoxy, di-(or tri-) hydroxy coumarin. The presence of two or more hydroxy groups is shown by the Ferric Chloride - Catechol reaction. The following formula can most

probably be assigned to it.



In other words itis most likely an isomer of Fraxin or Scopolin (11 & 12 & 13).

Although I have heen unable to prepare a crystalline sample of this glucoside, the following points as to its preparation were noted and may prove of value in any further work along the same line.

(1) The amount of material should be collected in the summer if possible, but material collected in winter may contain the desired glucoside.

(2) It is unnecessary to reduce the extract volumes at such low temperatures as 40°C, as the coumarin glucosides, including the new one, are thermostable, at least at 80°C.

(3) If the extracts are to be treated with yeast, thymol or other preservatives must not be used as the yeast is thereby inactivated and possibly destroyed.

(4) The use of yeast is undesirable as further impurities are thus introduced, and in the case of this particular glucoside, carbohydrates do not seem to be an important impurity.

(5) The property of fluorescence is a useful guide but should not be depended on too much as its intensity does not appear to vary as the concentration but rather as the hydrogen ion concentration and the presence of such solvents as alcohol and acetone. Although the alkaline solutions of the new glucoside are usually the most fluorescent, it is often fluorescent in acid

alcohol or acetone solutions. Very often the fluorescence increases with dilution.

(6) One should not decide on properties such as solubility, on impure preparations as the impurities very often give a totally different solubility. For example Charaux (6) found that the highly insoluble Fraxin was readily soluble in cold water when in the presence of a so called saponin. This may account for McCullagh's observation in regards to the solubility of the new glucoside in absolute alcohol.

(7) In preparation of coumarin glucosides, clarification with neutral lead acetate seems to be desirable, but one must boil up the filtered lead sulphide thoroughly as the glucoside is apparently very readily adsorbed by the lead sulphide, and thus a great portion of it lost.

(8) Lead acetate may be most conveniently prepared by either method of Hawk or Bertrand (4 & 5).

Note:- I first prepared basic lead acetate by the following method:-
500 cc. of a saturated solution of neutral lead acetate (saturated at a moderate temperature) were mixed in the cold with 100 cc. of ammonium hydroxide of a specific gravity of 0.96, in a well stoppered flask. After several days the basic lead acetate crystallized out and was filtered off & washed with a little water. Such a preparation dissolves in water without residue.

This latter method is unreliable and difficult and entirely unnecessary as the solutions prepared by the other methods (4 & 5) are entirely satisfactory and easy to prepare.

(9) In using basic lead acetate oneshould be very careful not to add an excess andto adjust the pH with ammonia until a maximum precipitate is produced. It is advisable to concentrate the extracts before this treatment.

(10) I have found that the acetone ether extraction method as described above gives a purer preparation sothat the use of basic lead is hardly indicated.

(11) Preparations tend to be difficult to dry and ~~the use of several~~ washings of absolute alcohol and ether and subsequent drying are required.

(12) There seems to be no danger of hydrolysis from the acetic acid introduced by the lead and thus neutralization is entirely unnecessary and extremely undesirable.

(13) The use of qualitative or quantitative Benedict's or Fehling's tests on hydroxy phenyl compounds is useless. Bertrand's method offers the best hope of good results.

(14) Estimation of double bonds by addition of iodine is hopeless, as already mentioned. Barger and Eaton (14) found that coumarins absorb varying amounts of iodine and never in molecular quantities, showing that one could not even allow for additional absorption as was done in the case of Copper reduction tests.

(15) Ziesel's method (7) of methoxy estimations seems to be all right for this work.

(16) Hydroxyl estimations might be done by acetylation (10) but of course would have to be done on the "Split-product".

(17) For polarimetric readings on the pure preparation, alcoholic solutions give the best results, being more clear.

(18) a. The glucoside appears to be readily hydrolyzed by dilute acids and alkalies and by emulsin.

b. The "Split-product" may be held in solution by steam -volatile impurity which should be removed after hydrolysis.

(19) Work up to the Present time seems to indicate that the hy- drolyzed product is soluble in ethyl acetate and ether and insoluble in cold water.

Although the work reported above is incomplete the results obtained will, at least, act as a guide to future work in the same field.

The author is very grateful to Professor A. T. Cameron, under whose direction this work was carried out, and to Dr. F. D. White for very great assistance and advice throughout its course.

REFERENCES.

(1) McCullagh, D.R. 1926 Trans.Roy.Soc.Canada Sect.V.331-337.
(2) Bourquelot.Em. 1901 Jorum.Pharm.Chim. 14 , 481.
(3) Benedict. 1909 Jour.Biol.Chem. 5, 485.
(4) Bertrand & Thomas 1920 Practical Biological Chemistry.
(5) Hawk & Bergeim 1927 IX. Practical Physiological Chemistry pg. 642
(6) Charaux. C. 1911 Journ.Pharm.Chim. 4, 248-250.
(7) Cohen, J.B. 1908 Practical Organic Chemistry 220-2.
(8) Benedict 1911 Jour.Am.Med.Ass'n. 57, 1193.
(9) Bertrand & Thomas 1920 Practical Biological Chemistry 61-74
(10) Cohen, J.B. 1908 Practical Organic Chemistry 222-4
(11) Armstrong, E.F. 1924 Carboydrates & glucosides 187-219.
(13) Meyer & Jacobson, Lehrbuch der Organischen Chemie 670-680
(12) Barger & Eaton 1924 J.Chem.Soc. 125, 2407-13.
(12) Czapek F. Biochemie der Pflanzen Dritter Band 474-490