INVESTIGATIONS ON THE BIOSYNTHESIS AND THE CHEMOSYNTHESIS OF GLUCURONIC ACID.

BY

WALTER R. ASHFORD

A THESIS SUBMITTED IN PARTIAL
FULFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF
MASTER OF ARTS
IN THE DEPARTMENT OF CHEMISTRY.

THE UNIVERSITY OF BRITISH COLUMBIA.

APRIL, 1941.

Accepted on behalf of the Department of Chemistry

Head of the Dept. of Chemistry.

---- TABLE OF CONTENTS ----

TOPIC	PAGE
ACKNOWLEDGMENTS	(ii)
INTRODUCTION	1
PHYSIOLOGICAL IMPORTANCE OF GLUCURONIC ACID	1
REVIEW OF METHODS OF SYNTHESIS	
(i) Biosynthesis	12
(ii) Chemical methods of synthesis	13
PROPERTIES OF GLUCURONIC ACID AND ITS COMPOUNDS	17
EXPERIMENTAL WORK	
(a) Biosynthesis	19
(b) Chemical synthesis	23
(i) Oxidizing agents	27
(ii) The effect of pressure	33
(iii) The effect of temperature	34
(iv) The effect of P^H	34
(v) Catalysts and their effects	37
(vi) Specificity of naphthoresorcinol test	39
(vii) Methods of separation	41
CONCLUSIONS AND DISCUSSION	43
BIBLIOGRAPHY	following 45

---- o o O o o ----

ACKNOWLEDGMENTS

I wish to express my thanks to Dr. R. H. Clark, under whose guidance this work was conducted, for his continued interest, and for his many helpful and stimulating suggestions.

Thanks are also extended to Mr. William Barclay and Mr. Gordon Hewitt, in conjunction with whom the work was done, to Mr. J. A. McCarter for valuable assistance and to Mr. G. Mathias and the Department of Bacteriology for their active co-operation.

---- o o O o o ----

INVESTIGATIONS ON THE BIOSYNTHESIS AND THE CHEMOSYNTHESIS OF GLUCURONIC ACID

INTRODUCTION

Glucuronic acid has always been of experimental value from a physiological point of view, but recently has taken on a new importance as a result of the discovery 1) of its function as a detoxification mechanism in the animal body

2) of its possible role in intermediary metabolism

and 3) of its use in the preparation of synthetic antigens.

To investigate these phases of the physiological chemistry of glucuronic acid, it is necessary to have a means of producing the substance in pure form, so that large-scale experimentation may be carried out. The first objective was therefore to devise a method, preferably chemical, by which glucuronic acid could be readily obtained in a pure state. The physiological aspects of the problem have not been attacked as yet, but it is hoped that this more important phase of the problem will be studied in the near future. In the following paper are given the results obtained from the attempt to synthesize glucuronic acid from such compounds of glucose as the oxime and the α methylglucoside.

I. PHYSIOLOGICAL IMPORTANCE OF GLUCURONIC ACID.

To show the necessity of obtaining a satisfactory method of making available a ready synthesis of glucuronic acid, for biochemical investigation, a short account of the physiological importance of the acid will be given.

Glucuronic acid was shown some years ago to be present in small amounts in blood, liver and urine, where it exists in the free or combined

form. In 1932 Quick (1) showed that the animal organism can synthesize glucuronic acid at the rate of one gram per hour, which finding led him to suggest that glucuronic acid may play a part in the metabolism of carbohydrates or at least has some important physiological functions.

(i) As a detoxification mechanism.

During the digestive process, certain nocuous substances are produced in the intestine. Others may result from metabolic processes in the tissues. For example, phenol may be produced from the breakdown of tyrosine and indole from the destruction of the tryptophane molecule. Some of these products are conjugated by glucuronic acid to form glucuronides, in which form they are excreted from the body. In this respect, glucuronic acid seems to have a great affinity for hydroxyl groups. Such substances as phenol and borneol are conjugated easily but terpenes are oxidized to corresponding alcohols and then conjugated. The action with hydroxyl groups is not specific as benzoic acid and "aspirin" are also readily conjugated.

It is of interest to note the types of conjugation which occur. They are 1) of the glucoside "ether" type such as that found in d-bornyl glucuronide, resulting in a compound which is non-reducing, 2) of the glucoside "ester" type such as benzoyl glucuronide which retains its reducing properties and 3) the type containing two glucuronic acid molecules, one attached to a non-sugar residue by a glucoside "ether" linkage and the other attached by an "ester" linkage. An example of this latter type is p-hydroxy benzoic diglucuronic acid.

(ii) Formation in the body.

Suggestions have been put forward to explain the formation of glucuronic acid in the body. Fischer (2) suggested two possibilities to explain the presence of glucuronic acid in the body. Glucuronic acid may be present naturally, or may be formed by some mechanism in the body and then conjugated with toxic substances introduced into or produced in the body. A second possibility suggested, was that toxic substances unite with glucose to form glucosides which are then oxidized to form glucuronides. The second possibility has been shown to be untenable as shown by Pryde and Williams (3) who fed phenyl α and β glucosides to animals and found that they were not converted into the corresponding glucuronides. Instead, they underwent hydrolysis and the liberated phenol was detoxicated and excreted as an ethereal sulphate. Further, it is known that glucuronic acid is not a normal oxidation product of glucose metabolism. Quick (4) has suggested that glucuronic acid is derived from body protein, basing his conclusions upon his findings that in the diabetic animal, glucuronic acid is produced from that portion of the protein molecule which would otherwise have gone to glucose. He believes that glucose and glucuronic acid have the same precursor in the body. In 1932 Quick (5) suggested that glucuronic acid is probably synthesized from short chain carbohydrate der ivatives. This suggestion has been expanded and proved with some degree of certainty by Lipschitz and Bueding (6) who by very careful experimentation found that the percentage of glucuronic acid synthesized in vitro by surviving liver slices was increased several hundredfold by the addition of dihydroxy acetone, pyruvic acid or lactic acid to the medium. They suggested that the carbonyl group of a triose probably conjugates with an alcohol and the resulting trioside is built up to the conjugated acid.

(iii) Factors influencing the production of glucuronic acid.

The velocity of production depends upon the nature of the substance to be conjugated. If a hydroxyl group has to be introduced by cell metabolism, as is the case with camphor, then the velocity of production of the conjugated product is slow. Quick (5) has shown that insulin can markedly increase, while acetoacetic acid as well as lactic acid, decidedly suppress the output of glucuronic acid. Lipschitz and Bueding (6) have made exhaustive tests on pure chemical substances which increase or decrease the production of glucuronic acid in surviving tissue slices. They found that the production of glucuronides was an oxidative process involving oxygen and that the oxidation process was catalyzed by a heavy metal, proof of which is contained in the fact that the oxidation process was completely inhibited by cyanide. Further, it was shown that an esterification of phosphoric acid with organic material is essential for the formation of glucuronic acid, because its production is sensitive to iodoacetate and to fluoride. Hemingway, Pryde and Williams (7) have studied the effect of cyanide upon conjugation in surviving tissue. Conjugation, they state, may depend upon the cellular integrity of the liver or upon the existence of a pre-formed enzyme. Potassium cyanide, added to artificially perfused liver outside of the body prevented the conjugation of phenol and glucuronic acid thus lending weight to the first suggestion. This, however, does not remove the possibility of an enzyme system taking part in the conjugation process. Cyanide may inhibit such an enzyme system if one is present.

The suggestion of Hemingway et al of the part played by an enzyme system is interesting in the light of the recent discovery by Fishman (8) of the enzyme glucuronidase, which he has shown to be specific for the hydrolysis of conjugated glucuronides.

(iv) Site of Formation.

Lipschitz and Bueding (6) have shown that conjugation takes place practically exclusively in the liver. Some conjugation may take place in the kidney.

(v) Glucuronic acid and the sex Hormones.

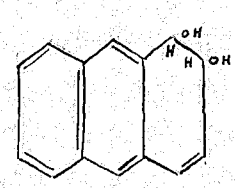
Several workers have shown that certain sex hormones are excreted as conjugated glucuronides. Among these are Cohen and Marrian (9) and Venning and Browne (10). The former have shown that oestriol is linked to glucuronic acid by a glucosidic linkage through the aldehyde group of the latter.

(vi) Possible relation of glucuronic acid to the cancer problem.

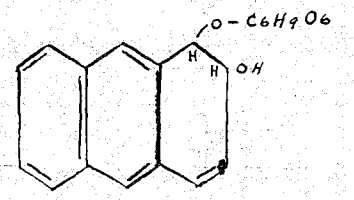
The discovery of the carcinogenic action of the sex hormones and their relationship, structurally, to known chemical compounds has been the inspiration for a wide variety of work upon these substances. It is now firmly established that the estrogenic hormones are growth-stimulating chemicals and that the female genital tissues are capable of responding to these chemicals during the greater part of the life of the individual. Work seems to indicate, too, that estrogenic hormones are at least indirectly responsible for mammary tumors. Their carcinogenic effect is directly proportional to their various physiological activities. Loeb (11) has presented pertinent evidence to show that some important relationship exists between the sex hormones and mammary cancer in the female body. He developed strains of female mice in 99% of which appeared spontaneous mammary cancer. Extirpation of the ovaries at a sufficiently early period reduced the cancer rate to zero. Lacassagne (12) produced mammary cancer in male mice, which would not normally have developed it, by weekly injections of crystalline female sex hormone preparations. Burrows (13) observed that mice injected

with oestrone developed cystic matsophathy, a condition regarded as a preparatory stage toward cancer. Other oestrogenic substances produced similar results.

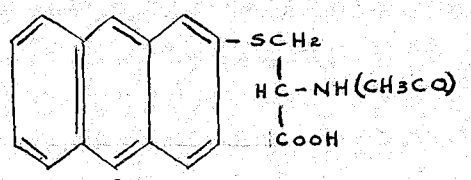
It has been shown many times (14) that various types of polycyclic hydrocarbons have carcinogenic properties. Whatever their direct or indirect role may be in the formation of malignant growths, it is clear that they have two characteristics in common. First they convert normal cells into new types, by an as yet unknown process. These new types are without a natural place in the animal organism. And secondly they are insoluble in body fluids. Studies have been made on such carcinogenic hydrocarbons as, 1,2,5,6, dibenzanthracene and anthracene itself with a view to determining what mechanism, if any exists in normal tissue to affect their detoxification. Boyland and Levi (15) have shown that normal tissues convert anthracene into at least three different compounds.



1.



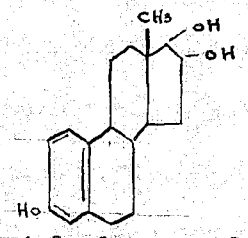
2.



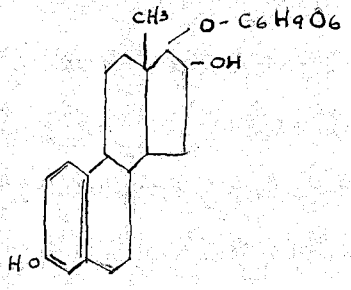
3.

No proof is offered as to whether these compounds represent successive steps in the metabolism of anthracene or whether they are formed independently of each other. Regarding the first of these mechanisms, the development of the glucuronide 1,2 dihydroxy 1,2 dihydroanthracene glucuronide, we have a reaction which has an analogy in the metabolism of sex hormones; that is the reaction of two adjacent secondary hydroxyl groups with glucurone to form a glucoside.

linkage rendering a glucuronide with water soluble properties. Oestriol



is so converted into oestriol glucuronide



and excreted as such from the animal body.

Many substances normally produced in the body are carcinogenic or are readily converted into carcinogenic substances. Methyl cholanthrene, a 5-methyl 1,2 benzanthracene containing a 5 carbon ring attached to the six and ten position was found to be very potent and is significant in view of its resemblance to the bile acids. Wieland and Dane (16) demonstrated that bile acids can be transformed into methyl cholanthrene by chemical means. It is interesting to note, too, that methyl cholanthrene resembles the sterols and the sex hormones in its five membered ring structure and it is conceivable that methyl cholanthrene may be a degradation product of these substances.

Acetyl choline, a substance set free by the so-called cholinergic nerves of the sympathetic nervous system has been found highly carcinogenic. If administered to animals subcutaneously it produces osteo sarcoma.

Work has been done to determine the relationship between the action of oestrone and the female sex hormones generally, and the direct carcinogenic

action of hydrocarbons. Does oestrone act by direct carcinogenesis or is its action due to its physiological function under abnormal conditions? It is claimed that the mechanism of the production of carcinoma by carcinogenic hydrocarbons and by oestrone is entirely different. It has been shown that oestrone does not act through its polycyclic structure. Stilbestrol, an oestrogenic substance of greater power than oestrone has been prepared by Doads, Goldberg, Lawson and Robinson (17). This substance acts like oestrone, is highly carcinogenic and yet lacks the polycyclic structure. Does this necessarily show that it is the polycyclic structure of hydrocarbons or of sex hormones which causes carcinogenicity? And does it show that the sex hormones are different in their action to that of the hydrocarbons? It is suggested that it does not, because so many substances of dissimilar structure have high carcinogenic activity. Amino, azo and nitro compounds have been shown to be carcinogenic by Waters (18) and Shear (19). When so many diverse substances, organic and inorganic, have been shown to be carcinogenic, it would seem that chemical structure is not primarily associated with carcinogenesis.

A mechanism of carcinogenesis has been suggested. The work of Warburg (20) has shown that malignant tissue differs from most normal tissues in producing more lactic acid than it is able to oxidize. Keilin (21) has shown that the respiratory system of most cells can be divided into three parts:

1) Indophenol oxidase, which activates oxygen.
2) Cytochrome, a reversibly reducible pigment which acts as an oxygen carrier.
3) The dehydrogenases, e.g. lactic acid dehydrogenase which activates lactic acid so that it can reduce cytochrome or methylene blue.

The respiration of tumors is greatly increased by the addition of p-phenylene diamine showing that there is ample indophenol oxidase or "respiratory enzyme". Cytochrome has been found in most malignant tissues examined. Therefore the defect of malignant tissue may be a deficiency in dehydrogenases. Carcinogenic hydrocarbons have been shown to inhibit the dehydrogenases, which, when absent allow the local accumulation of lactic acid. Further, it is known that tumor cells survive and grow in media containing lactic acid, whereas normal cells may be inhibited by the presence of this substance. Tumor tissues have a mixed metabolism and produce lactic acid both in the presence and absence of oxygen. Normal tissues produce little or no lactic acid aerobically and must have free oxygen to live. If cells in a certain tissue should become malignant due to say the presence of excess sex hormones, they would be able to grow and reproduce to the disadvantage of surrounding normal cells.

Whether chemical carcinogens, including the sex hormones, act as such, or whether they undergo chemical changes in the tissue before they acquire carcinogenic properties is not known but there should be some mechanism in the body which removes toxic substances and excess sex hormones before they become carcinogenic. At any rate, the body contains in its tissues definite growth activators and inhibitors. Murphy and Sturm (22), (23) have isolated from placenta and embryonic skin, substances which definitely inhibited transplanted and tumorous growth. The mammary gland in the pre-lactating stage yielded a fraction which gave a marked stimulation to tumor growth. It is possible that glucuronic acid may have an important role here. In the case of oestriol, a glucurone-oxidase enzyme system has been observed by Fishman (8), (24) which is responsible for the conjugation of oestriol with glucurone forming a water-soluble complex, and thus making possible

its elimination from the body. The same or a similar system may exist affecting the hydrolysis of the glucuronide. If, for some reason, tissue cells can no longer conjugate the insoluble hormones to their water-soluble form, or if the enzyme balance is upset so that hydrolysis of the glucuronides occurs at a greater rate than their synthesis, then a preponderance of insoluble carcinogenic compounds will result which may have their effect upon the respiratory system of the cell, as suggested above. This suggestion is put forward only with reference to tumors which may be caused by an over-accumulation of female sex hormones in the animal body.

Much evidence has been collected to show that carcinogenic substances may be anti-carcinogenic under certain circumstances. Haddow and Robinson (25) found that carcinogenic hydrocarbons inhibited the growth of Jensen and Walker tumors. Nitta (26) used sex hormones to inhibit tumor growths. If, aswas suggested before, the enzyme system balance has been upset, so that hydrolysis of conjugated glucuronides occurs at a greater rate than their synthesis, then carcinogenic agents may act anti-carcinogenically by restoring the enzyme balance.

Work on this phase of the problem has not been attempted, but several approaches to a solution might be applied.

1) Studies might be made on the enzyme system with regard to its ability to synthesize or hydrolyze glucuronides and on the effect on the reversibility of the reaction of various activators and inhibitors.

2) By the method of tissue culture to try to produce malignant cells in vitro by means of carcinogenic agents including sex hormones and to study the effect of glucuronic acid and the enzyme glucuronidase upon the production of such a system.

3) Investigate the production of cancer carcinogenically on the live

animal in two cases--first where glucuronic acid is administered along with the carcinogen and second where the carcinogenic agent is applied alone. The effect of glucuronic acid might be tried also upon a strain of mice with a known high incidence of cancer.

4) Study the effect of activators and inhibitors on the animals capacity to produce the enzyme glucuronidase.

It is not to be concluded from the above remarks that it is believed that glucuronic acid will solve the cancer problem. The factors concerned are many and involved. For example, little is known concerning the role of the pituitary gland and its secretions, but they must have an important function, controlling as they do the whole endocrine system of the body. It seems possible, however, that glucuronic acid acting in a detoxification role may be a link in the chain of events.

II. REVIEW OF METHODS OF SYNTHESIS.

(i) Biosynthesis

Quick (27) has been a pioneer worker in the biochemical production of glucuronic acid. He condemns menthol as a glucuronogenic drug when fed to rabbits due to difficulty in administering the substance and the labor involved in obtaining large quantities of glucuronic acid. Dogs are able to destroy menthol or conjugated menthol. The method finally evolved by Quick was to feed five grams of borneol daily to each of several dogs; to isolate the conjugated glucuronide from the urine as the zinc salt and then to prepare the free acid from this salt. By this method, Quick was able to obtain one gram of zinc salt for every gram of borneol fed and a final yield of glucuronic acid of over 80% of the theoretical.

In 1911, Bang (28) prepared menthol glucuronic acid from rabbits. It consisted in feeding rabbits two grams of menthol by stomach tube, collecting the urine for twenty-four hours and extracting the ammonium salt by half-saturation with ammonium sulphate.

Recently, this method has been modified by Williams (29) so that he obtained a yield of 1.4 grams of the crude ammonium menthol glucuronate for every gram of dl-menthol fed. Williams (30) investigated the use of other glucuronogenic drugs and gives the following relative values of materials for conjugation with glucuronic acid in the rabbit.

l-menthol	48% conjugation
dl-menthol	59% conjugation
d-menthol	70% conjugation

Concerning the relative merits of the preparations of glucuronic acid from dogs and from rabbits as presented by Quick and Williams, respectively, the following may be said.

1. The method using dogs has the advantage of ease of administration of the glucuronogenic drug.

2. Larger quantities can be produced by using dogs as they can receive borneol every day with no apparent ill-effects.

3. Quick,using dogs and borneol reports a better yield than does Williams using rabbits and menthol.

4. Quick's method has the advantage of ease of separation of the zinc salt from the urine. The separation is more complete than in Williams' separation using ammonium sulphate.

5. Rabbits are cheaper to buy, more cheaply fed and easier to look after than dogs. Larger numbers of them can be kept.

(ii) Chemical methods of synthesis.

a) oxidation of Glucose.

Nef (31) reported the following products as a result of the oxidation of d-glucose in the presence of copper hydroxide in alkaline solution. 114 gr. d-glucose gave 3.83 gr. CO_2, 14.71 gr. glyceric acid, 30 gr. trioxybutyric acid and 30 gr. of hexonic acids. Acids formed varied with the concentration of alkali present.

Killiani (32) oxidized glucose (100 gr.) with nitric acid (.8 vol-1.2) for fourteen days and obtained 10.1 gr. "of what is apparently calcium glucuronate". Killiani makes two important comments: 1) only 1.333 atoms of oxygen per mole of glucose was used instead of the two atoms calculated for the reaction; hence the regeneration of nitric acid which took place must play a material part in the reaction. 2) Glucuronic acid itself is very stable towards nitric acid (d = 1.2) at room temperature, for the calcium salt precipitated from water solution contained only a minimal amount of calcium saccharate.

Jolles (33) oxidized dextrose (2% solution) with H_2O_2 (12 vol. %) at 37° for 144 hours. The unused glucose was removed with yeast. Glucuronic acid was removed as the lead salt, the yield being described as poor.

b) Oxidation of Glucosides.

Glucose and glucosides were suggested as possible primary compounds for the preparation of glucuronic acid by Fischer and Piloty (34). Fischer suggested that one source of glucuronic acid in the body might be through the oxidation of glucosides. Toxic substances introduced into or formed in the body united with glucose to form glucosides. Subsequent oxidation produced glucuronides. More recent work, as indicated above seems to show that this is not the case.

Smolenski (35) attempted to apply Fischer's idea to a method for the chemical synthesis of glucuronic acid. ∝ Methyl glucoside oxidized by means of bromine and sodium carbonate or by hydrogen peroxide in the presence of ferric hydroxide produced methyl glucuronide in yields up to 30%. Isolation was by means of the brucine salt.

Jackson and Hudson (36) studied the cleavage of the carbon chain of glucosides by oxidation. The oxidation of ∝ methyl d-glucoside with barium hypobromite produced a dibasic acid which was isolated as its crystalline strontium salt. Yields of 65-70% were obtained. Periodic acid as an oxidizing agent produced similar results.

The oxidation of maltose as a natural glucoside by Glattfield and Hanke (37) gives further evidence of the destruction of the glucose molecule on oxidation. Using hydrogen peroxide as an oxidizing agent, a great variety of substances was obtained including formic acid, glucosido acids, glycollic acid and oxalic acid.

Craik (38) studied the mechanism of the oxidation of typical carbohydrates with hydrogen peroxide. Maltose was not attacked by hydrogen peroxide except in the presence of ferrous sulphate. When oxidation did occur, maltose was attacked first at the reducing group giving a maltobionic acid which, being fairly strong, caused hydrolysis of the unchanged sugar. Subsequently, oxidation of the fragments occurred giving a great variety of products.

Bergmann and Wolff (39) oxidized α menthol glucoside with bromine in the presence of pyridine and normal sodium hydroxide. A yield of 7.5% of α menthol glucuronide was obtained.

c) Reduction of the lactone of saccharic acid.

Fischer and Piloty (34) obtained glucuronic acid in yields up to 10% by reducing the lactone of saccharic acid with sodium amalgam.

d) Electrolytic method.

Leutgoeb and Heinrich (40) employed an electrolytic method for the oxidation of α methyl glucoside. The ultimate oxidizing agent was hydrogen peroxide. No catalyst was employed. Extraction was by means of the cinchonine salt. Eleven atmospheres pressure was employed. The yield obtained was in the neighbourhood of 20.2%.

e) Pure chemical synthesis.

Stacey (41) devised a method for the oxidation of the sixth carbon atom of glucose after protecting the remaining carbon atoms with acetyl groups. Glucose was converted into 6 trityl penta-acetyl glucose. The trityl group was then removed and the resulting penta-acetyl glucose oxidized at the sixth carbon atom with potassium permanganate. The yield obtained was 20% over all.

Zervos and Sessler (42) started with acetone glucose and converted it

to 1,2 acetone 3,5 benzylidine ∝, d-glucofuranose. Oxidation by cold alkaline potassium permanganate produced acetone-benzylideneglucuronic acid which when heated to 100°for one hour in 50% alcoholic hydrochloric acid solution produced d-glucurone. Five grams of acetone glucose produced .9 gr. glucurone.

Consideration and comparison of the above methods indicate that none is highly satisfactory. Yields are small in all cases. The last two mentioned, those of Stacey and Zervos and Sessler, are beautiful examples of organic synthesis, but much too involved to be considered as practical methods for the commercial preparation of the substance. The method involving the electrolysis of methyl glucoside requires expensive apparatus, while the yield is relatively low. Smolenski's method involving the oxidation of methyl glucoside with hydrogen peroxide plus ferric hydroxide with the production of a 30% yield of glucuronic acid is interesting. Unfortunately only the abstract of the paper is available and none of the details of the method employed are known.

III. PROPERTIES OF GLUCURONIC ACID AND ITS COMPOUNDS

COMPOUND	OPTICAL ROTATION	MELTING POINT	REFERENCE
l-glucurone		169-172°	34
d-glucurone	$[\alpha]_D^{23} = +18.55$		43
d-glucuronic acid — after 3 min.	$[\alpha]_D^{24} = +16.05$ in H_2O		
d-glucuronic acid — after 3 hrs.	$[\alpha]_D^{24} = +36$ in H_2O		
zn. dl-borneol glucuronate .2 H_2O		Decomp. 206°	
l-borneol glucuronic acid (anhyd.)	$[\alpha]_D^{20} = -69.03$ in H_2O	162-3°	
d-borneol glucuronic acid (anhyd.)	$[\alpha]_D^{20} = -37.02$	164-5°	
β-triacetyl glucurone	$[\alpha]_D^{23} = +84.1$ in $CHCl_3$	194-195°	44
α-triacetyl glucurone	$[\alpha]_D^{24} = -203.6$ in $CHCl_3$	110-112°	44
diacetyl chloro-glucurone	$[\alpha]_D^{22} = +95.5$ in $CHCl_3$	107.5-108.5°	45
Diacetylglucurone — after 2 min.	+1.60		
Diacetylglucurone — after 25 min.	+ 0.85	130-131°	45
Silver glucuronate (in H_2O)	$[\alpha]_D^{23} = +19.2$		46
Methyl glucuronate (in H_2O)	$[\alpha]_D^{23} = +44.6$		46
β tetraacetyl glucuronic acid methyl ester.	$[\alpha]_D^{24} = +8.7$ in $CHCl_3$	178°	46
α tetraacetyl glucuronic acid methyl ester.	$[\alpha]_D^{24} = +98.0$ in $CHCl_3$	111-112°	46
l-chloro-triacetyl glucuronic acid methyl ester.	$[\alpha]_D^{21} = -16.7$ in $CHCl_3$ C = .6%	150.5-151.5°	46
Diacetyl methyl glucoside of glucurone.	$[\alpha]_D^{23} = +112.5$ in CHCl C .6%	110-111°	47
Triacetyl methyl glucoside of glucuronic acid methyl ester.	$[\alpha]_D^{23} = +54.0$ in $CHCl_3$ C = .6%	118°	47

COMPOUND	OPTICAL ROTATION	MELTING POINT	REFERENCE
Diacetyl p-nitrobenzyl glucoside of Glucurone.	$[\alpha]_D^{24} = +39.9$ in $CHCl_3$ C = 1%	133-134°	47
Triacetyl p-nitrobenzl glucoside of glucuronic acid methyl ester.	$[\alpha]_D^{26} = -57.8$ in $CHCl_3$ C = .6%	175-176°	47
α Triacetyl chloroglucuronic acid methyl ester.	$[\alpha]_D^{24} = +168.7$ in $CHCl_3$ C = .5%	99-100°	48
α triacetyl bromoglucuronic acid methyl ester.	$[\alpha]_D^{24} = +198.0$ in $CHCl_3$ C = .5%	104-105°	48
β Methyl glucoside of Triacetyl glucuronic acid Methyl ester.	$[\alpha]_D^{25} = -28.9$ in $CHCl_3$ C = .7%	149-150°	48
β p-Nitro benzyl glucoside of Glucuronic acid methyl ester.	$[\alpha]_D^{23} = +63.2$ in H_2O C = 1%	167-168°	48
1,2,3,4 Diacetone galactose 6 2,3,4 Triacetyl glucuronide Methyl Ester.	$[\alpha]_D^{24} = -68.0$ in $CHCl_3$ C = 2.0%	112.5-114°	49
Galactose 6, β-Glucuronide	Initial $[\alpha]_D^{24} = +9.4$ Final-2Hrs. $[\alpha]_D^{24} = -7.3$ C = 1% in H_2O	116-119°	49
Methyl Ester of Galactose 6β Glucuronide.	Initial-6 Min. $[\alpha]_D^{24} = -2.90$ FInal $[\alpha]_D^{24} = -9.1$ C = 4.0 % in H_2O	119°	49
Heptaacetyl Methyl Ester of Galactose 6β Glucuronide.	$[\alpha]_D^{25} = -17.5$ in $CHCl_3$ C = 3%	202-203°	49
Trimethyl glucurone.	$[\alpha]^{26°}_{5461} = +197.5$ C = .76 in H_2O.	131-132°	50

IV. EXPERIMENTAL WORK.

a) Biosynthesis.

Preparatory to attempting a chemical synthesis, it was necessary to obtain a pure sample of glucuronic acid or of one of its compounds. For this purpose the biosynthesis of Williams (29) was followed with certain modifications.

Difficulties encountered in this method and the modifications applied are as follows. Rabbits (female) weighing between $2\frac{1}{2}$ and 3 kg. were used. They were housed in a cage having a wire top and bottom. The cage was placed in a wooden stand having a galvanized iron funnel-shaped bottom, of the size of the bottom of the cage. Urine collections were therefore easily made in a large erlenmeyer. A fine copper screen wire was placed between the cage bottom and the funnel to prevent food or faeces from entering the urine.

As neither d or dl-menthol was available, l-menthol was used throughout all experiments. Previous workers have recommended feeding one gram of glucuronogenic drug per kg. of body weight. It was found that more than this could be given in the case of l-menthol. Rabbits weighing 2700 Gr. received 4 gr. of l-menthol at each feeding. Feedings in our case took place once a week but could be increased to once every three days.

To administer l-menthol to a rabbit it is necessary to anaesthetize the animal and then to use an enema tube to place the compound in the stomach. The animal was best held by placing it in a large round tin about 18 inches high and 8 inches in diameter, padded on the inside. Ether was administered by an aspirator. Care should be taken to use a good grade of ether and one free of peroxides. The ether was tested for peroxides by acidifying with acetic acid, adding potassium iodide and starch and heating. If present,

peroxides free the iodine and the starch solution is colored blue. If peroxides are present, they can be removed by distilling the ether over water and copper wire into a brown bottle containing a copper coil, the bottle being kept full to exclude the presence of air. The rabbit's mouth was held open by a bone spatula through which a hole had been bored to permit the entrance of the enema tube. If the rabbit is held upright, there is little possibility of forcing the tube down the trachea even when the rabbit is under ether.

The rabbit should be starved for 24 hours before being given the menthol. Following Williams' suggestion the menthol was made into an emulsion with about 30 c.c. of warm water. Difficulty was experienced at this point. The rabbits came out of the ether within five minutes of the administration of the menthol. Within one half hour after the rabbits came out of the ether, they lapsed into a state of coma, which lasted for about three hours in the case of one rabbit and two hours in the case of the others. The animals appeared to be groggy and sleepy. All voluntary action was inhibited and reflex action of the eye was stopped. The rabbits appeared to be extremely hungry especially when coming out of the coma. No ill effects were suffered, as far as could be determined by observation. This phenomenon happened at each feeding and was thought at first to be due to the ether used, but this possibility was removed by freshly distilling the ether and thus removing peroxides.

An attempt was made to discover the cause of the phenomenon just described. It was thought that it might be due to shock caused by removal of glucose from the blood stream of the animal. It was thought too that information on this point would indicate whether or not body glucose is used by the animal in synthesizing glucuronic acid.

The following experiment was carried out using three rabbits.

Rabbit No. I.

10 c.c. of blood were withdrawn from the marginal vein. Four grams of l-menthol were fed by the method described above. It was noted that this animal did not go into a state of coma as soon as those from which no blood had been withdrawn, but after one and one half hours it lapsed into a condition resembling death. Even the heart was slowed down and body heat decreased. At this point an additional 5 c.c. of blood were withdrawn from the marginal vein. Later, when the rabbit had been in the state of coma for about two hours, it was given 3 gr. of glucose in water by stomach tube. This was apparently without effect, as the animal did not completely recover for another four hours.

Analyses for blood sugar were made on the two samples, by the method of Somogyi-Shaffer-Hartmann (51). No significant difference was found in the two samples. It was concluded, therefore, that the coma was not caused by the shock of depleting the blood of glucose. Menthol apparently does not unite with glucose first to form a glucoside as was suggested by Fischer.

Rabbit No. 2

4 gr. of l-menthol and 4.6 gr. of glucose in a water emulsion were administered to a rabbit weighing 2800 gr. This animal did not go into a coma as rapidly and recovered more quickly than in previous times when no glucose was given. This was not interpreted as supplying evidence that body glucose went to the formation of glucuronic acid.

Rabbit No. 3.

The effect of borneol on rabbits was tried. If borneol could be used it would be advantageous, because of the ease of separating out the insoluble zinc salt. Accordingly, 3 gr. of l-borneol dissolved in olive oil

was fed to one rabbit. The effect was different than in the case of menthol. The animal did not go into a state of coma, but suffered intestinal spasms which must have been painful. The effect produced by borneol lasted much longer than that produced by menthol three days being required for complete recovery. During this time, the rabbit suffered a loss of appetite and lost much weight. No conjugated glucuronide was recovered from the urine. It was decided that either borneol is metabolized or that it passes through the rabbit unchanged, and that borneol is not a satisfactory glucuronogenic drug for rabbits.

An attempt was made to prevent the oncoming of the state of coma after administration of menthol. It was found that 4 gr. of l-menthol could be administered to a rabbit of 2.5-3.0 kg. without producing the after effects of the coma, if the menthol was dissolved in olive oil. Slight dizziness did occur in some cases when olive oil was used, but nothing like that produced by menthol alone in water. All latter experiments were cafried out, using olive oil as a solvent for menthol. The only explanation for the effect of the olive oil is one of slower absorption. The urine should be collected over a longer period of time when olive oil is used.

The method of treatment of urine was the same as given by Williams (29). Yields of crude ammonium menthol glucuronate are reported by the latter as being 1.4 gr. of crude glucuronate for every gram of d-l menthol fed, which conjugates to the extent of 59%. Over twelve l-menthol administrations yielded one gram of crude ammonium menthol glucuronate per gram of l-menthol fed, which corresponds to a 48% conjugation of l-menthol as reported by Williams. Yields were not affected by using olive oil in the administration of the menthol.

Living and Post-mortem examination of the rabbits.

At the end of a five month period which included fifteen administrations of l-menthol, the rabbits were in excellent health and appeared to have suffered no ill-effects.

Post-mortem and histological examination was carried out. Parts examined were oesophagus, stomach, small and large intestine, liver, lungs and kidneys. Gross examination showed one rabbit to have a haemorrhage of the lungs. This was probably not primarily due to the animal having been fed menthol. The liver showed one white area on the edge of one lobe. This is the first sign of liver disintegration. The inside wall of the stomach was very carefully examined for the presence of stomach ulcers. One area which very much resembled the so-called chronic or round ulcer was found, about the middle of the stomach.

Conclusions concerning the Biosynthesis.

Exact methods of procedure for the production of glucuronic acid from rabbits has been outlined. The biochemical method using either dogs or rabbits offers probably the best means now available of synthesizing glucuronic acid.

It is produced relatively cheaply and in good yield, and is separated from the urine as a stable compound.

The process of removing the glucuronic acid from the secreted compound offers no difficulties and can be carried through practically quantitatively. The glucuronogenic drug is recoverable and can be used over again.

b) Chemical synthesis.

In this investigation it was proposed to prepare glucuronic acid from glucose by oxidation after first protecting the aldehyde group by the formation of some condensation product, such as the phenyl hydrazone,

oxime or glucoside, The primary alcohol group would then be oxidized to a carboxyl group and the resulting compound hydrolyzed to give the aldehyde group back again, thereby forming glucuronic acid.

The glucose phenylhydrazone was made according to the method of Jacobi (52). Glucose 2,4 dinitro phenylhydrazone was also prepared. Neither hydrazone was found to be satisfactory for oxidation to glucuronic acid due to their instability.

Glucose oxime was prepared according to Wohl (53) by Gwyn (54).

α Methyl glucoside was prepared by the method of Patterson and Robertson (55). This compound has been shown to be very suitable for the purpose, being stable in so far as the linkage between the methyl alcohol and the glucose molecule is concerned.

The natural glucoside maltose was given consideration as a possible starting point.

Borneol glucoside was thought to provide a good starting point, as by keeping zinc ion in the oxidation mixture it would be possible to precipitate out zinc borneol glucuronate as soon as any borneol glucuronide was formed. The compound borneol glucoside was prepared accordingly. The tetra-acetyl brom derivative of glucose was first prepared according to the method of Koenigs and Knorr (56). This product was then condensed with borneol in the presence of silver carbonate by the method of Fischer and Raske (57).

The following modifications to the method of synthesizing borneol glucoside were made. The procedure for making tetraacetyl brom glucose requires at least ten hours for completion according to the method above. If ether is added to the reaction to prevent the contents from becoming too viscous a mercury seal stirrer can be used to keep the reactants in motion.

Thus, place glucose, glass beads, (small) and acetyl bromide in a claisen flask with the sidearm removed. Add sufficient ether to prevent the reaction mixture from becoming too viscous. Into the straight arm of the claisen flask insert a mercury seal stirrer and into the other a calcium chloride tube to permit escape of hydrogen bromide and prevent the entrance of water vapour. The flask is then completely covered to prevent entrance of light. The temperature should not go above 5°C. If the reaction becomes too vigorous as indicated by the evolution of hydrogen bromide it can be slowed down by lowering the temperature of the mixture. Stirring is continued until all the glucose has gone into solution which should be the case in about half an hour. The yield in this step is about 60% and in the second step about 50%, so that the overall yield is only 30%. Difficulty was experienced in oxidizing the compound, partly due to its sparing solubility in water. In view of this and the difficulty involved in its preparation, coupled with a low yield, it was decided not to proceed further in the study of borneol glucoside as a possible oxidizable substance.

The investigation was carried out along theoretical lines in an endeavour to discover the value of different oxidizable substances and different oxidizing agents and to discover the effect of pressure, temperature and various catalysts. Experiments on these phases of the problem involved the use of Tollen's naphthoresorcinol test as modified by Maughan, Evelyn and Brown (58) and more recently by Mozolowski (59) and were carried out on a small scale. When the problem of separation was attempted, difficulties were encountered. If glucuronic acid is present even in fairly complex mixtures, it should be relatively simple to separate. Therefore, inability to separate yields indicated by maphthoresorcinol

led to a study of the specificity of the reagent.

The Klett-Summerson photo-electric colorimeter was standardized as follows. Solutions of purified ammonium menthol glucuronate (M.P. 185° with decomp.) were made up so that they contained the equivalent of .01, .02, and .05 mg. of glucuronic acid per 2 c.c. of solution. 2 c.c. portions of these solutions were taken and to them were added 2 c.c. portions of a 0.2% solution of naphthoresorcinol reagent and 2 c.c. portions of concentrated hydrochloric acid. The mixtures were heated on a water bath for thirty minutes and then cooled in ice water for ten minutes. A blank test consisting of 2 c.c. of distilled water, 2 c.c. of conc. hydrochloric acid and 2 c.c. reagent should be run at the same time. After cooling for ten minutes the coloring matter was extracted with three 5 c.c. portions of ether containing about 15% absolute alcohol. Volumes were made up to the mark in calibrated Klett colorimeter tubes.

Colorimeter readings obtained with different samples of naphthoresorcinol are as follows.

TABLE I.

	Glucuronic Acid/2 c.c.	Klett
1	.01 mg.	32.5
	.02	55.0
	.05	82.0
2	.0125 mg.	45.0
	.025	36.4
	.05	65.0
3	.025 mg.	45.0
	.04	58.0
	.05	68.0

It is seen, therefore, that supposedly identical solutions of naphthoresorcinol produced different colorimetric readings, although the three curves produced are parallel lines. It was necessary to recalibrate the instrument for each new solution of reagent.

(i) Oxidizing Agents.

Several oxidizing agents were tried. Hydrogen peroxide was tried first because of the ease with which any excess can be removed and because of its gentle oxidizing power. Any excess hydrogen peroxide was removed from the oxidation mixture before the naphthoresorcinol test was applied. The effect of hydrogen peroxide on the test was determined. Results are as follows.

TABLE II.

	Solution Tested	Result
1.	Dilute H_2O_2 solution	green color extracted in ether layer.
2.	Solution of glucuronic acid	reddish purple in ether layer.
3.	10 drops 30% H_2O_2 plus 2 c.c. of (2) above	dirty green color in ether layer.

It was concluded therefore that hydrogen peroxide destroys the naphthoresorcinol test.

The presence or absence of hydrogen peroxide in reaction mixtures was determined by the following test. 1 c.c. of the solution to be tested was added to 1 c.c. of dilute potassium dichromate solution and to the mixture was added 2 or 3 drops of one normal sulphuric acid. The solution was immediately extracted with ether. A blue color in the ether layer indicated the presence of hydrogen peroxide.

Methods of destroying excess hydrogen peroxide were studied. Since an aqueous solution of hydrogen peroxide is stabilized toward heat by the presence of acids and since oxidation produces acidic substances, boiling will not remove excess hydrogen peroxide. Distillation of the aqueous part of an oxidation mixture did not remove all of the excess oxidizing agent even when the solution was taken down to a syrup. Hydrogen peroxide can be destroyed by manganese dioxide in neutral solution. The method followed in reaction mixtures to decompose hydrogen peroxide was to adjust the solution to $P^H 7$ with a suitable dilute base and then to add excess manganese dioxide which was later removed by filtering. The action here is catalytic. A second catalytic method of removing hydrogen peroxide was to add platinized asbestos to the solution removing the latter by filtration.

Hydrogen peroxide was used to oxidize methyl glucoside, glucose oxime (by Gwyn 54) and maltose. The greatest colorimetric reading was obtained when hydrogen peroxide was used in the ratio of four of oxidizing agent to one of the oxidizable substance.

A typical experiment performed is as follows. 5 grams of α methyl glucoside were dissolved in 80 c.c. of water. To the solution was added 5 c.c. ferric chloride solution (.0001 gr./c.c.) and 7 c.c. of 30% hydrogen peroxide. The mixture was placed in a pressure flask, sealed, and maintained at a temperature of 50°C for ninety-five hours. A test by the photo-electric colorimeter, using naphthoresorcinol, showed a 10.78% conversion to glucuronic acid. 10 c.c. of the mixture was titrated with .1126 N sodium hydroxide and found to require 31.6 c.c. showing that the entire reaction mixture contained .0331 equivalents of acid. Next, 10 c.c.

of the mixture was distilled under vacuum and the distillate titrated with .1126 N sodium hydroxide. 20.6 c.c. of the base were required showing the presence of .0214 equivalents of volatile acids. Non-volatile acid was therefore equal to .0117 equivalents. .0258 equivalents of ∝ methyl glucoside were used in the reaction. These results indicate that the glucose molecule is split probably at carbon atom three, with the formation of formaldehyde and formic acid.

A typical experiment employing maltose is as follows. 12 gr. of maltose were dissolved in 50 c.c. of water. To this solution was added 10.2 c.c. of 30% hydrogen peroxide and 5 c.c. ferric chloride (.0001 gr./c.c.). The mixture was placed in a pressure flask, sealed, and kept at 50°C for 30 hours in a constant temperature oven. A test with naphthoresorcinol indicated the presence of .555 gr. of glucuronic acid.

Conclusions concerning hydrogen peroxide.

1) H_2O_2 has a distinct advantage in being cheap.

2) Any excess is easily removed.

3) If used at room temperature its action is slow. If used at elevated temperatures, other reactions are aided as well as the oxidation of the primary alcohol group.

4) Apparently there are several competing reactions, namely:

a) Oxidation of the 6th carbon atom.

b) Breakdown of the glucose molecule at the third carbon atom.

c) Possible hydrolysis of ∝ methyl glucoside by acids formed by oxidation with hydrogen peroxide.

Use of barium iodide and iodine as an oxidizing agent.

Goebel (60) oxidized glucose with iodine in an alkaline solution and

obtained a 91 % yield of gluconic acid. It was thought that the same method might be applied to the oxidation of ∝ methyl glucoside to obtain methyl glucuronide. The procedure was as follows. 4.5 gr. of ∝ methyl glucoside were dissolved in water. To this solution was added 25 gr. of barium iodide (Merck) and 25.4 gr. iodine and the whole made up to 350 c.c. The mixture was warmed to 50°C and 500 c.c. of .4 N barium hydroxide was added from a dropping funnel over a period of fifteen minutes at a constant rate of flow the solution being mechanically stirred during this operation. The solution was stirred for fifteen minutes after the addition of the barium hydroxide and was then acidified with 9.25 c.c. conc. sulphuric acid. 75 gr. basic lead carbonate were added immediately with vigorous mechanical stirring, which was continued until the solution reached a P^H of 5 when the precipitate was allowed to settle, then filtered off and washed several times with water. The resulting filtrate gave a good test with naphthoresorcinol reagent. The filtrate was evaporated down to a small volume under vacuo whereby excess iodine was removed, and a yellow precipitate of lead iodide settled out which was filtered off. Dilute sulphuric acid was added to precipitate the lead which was removed by centrifugation. Any hydrogen iodide left was removed by silver sulphate and excess silver by hydrogen sulphide. Sulphate ion was removed by barium hydroxide and the excess barium by means of carbon dioxide. This ion-free solution at this stage gave a good naphthoresorcinol test. The solution was concentrated to a small volume and then to it was added several volumes of 95% alcohol which caused a white precipitate to settle out.

Iodine was tried as an oxidizing agent in the belief that a stronger

and faster oxidizing agent than hydrogen peroxide would act on the sixth carbon atom of glucose before attacking the rest of the molecule. By carefully controlling the time of oxidation, and the temperature of the reaction, and removal of oxidizing agent, it was hoped to stop the reaction at the crucial moment.

Accordingly, the above procedure was modified as follows. 5 grams of ∝ methyl glucoside were dissolved in 50 c.c. of water and to the solution was added 5 c.c. of ferric chloride (.0001 gr./c.c.). 3.2 gr. of finely pulverized iodine were added and just enough barium iodide (Merck) to initiate solution of the iodine. The beaker was placed on an ice bath and 15.4 gr. of barium hydroxide in 400 c.c. of water added with constant stirring over the course of three days. At the end of this time a small excess of barium bydroxide was added to decolorize the solution. A precipitate (probably barium iodate) was filtered off and to the filtrate was added excess lead acetate to remove lead iodide which was separated by filtering. The remainder of the iodine was removed with silver acetate. Lead ion was removed with hydrogen sulphide and barium ion with excess sulphuric acid. At this point the solution was hydrolyzed for three hours, after which the excess sulphuric acid was removed with lead acetate and the lead ion with hydrogen sulphide. Tests with naphthoresorcinol produced a deep red color. The solution was evaporated to a thick syrup which on warming slightly, crystallized to a solid mass. These crystals were taken up in hot 95% ethyl alcohol. A small white residue did not dissolve and was filtered off. This remained unidentified. The solution, on standing, produced large prismatic needles. Recrystallization to a constant melting point of 55°C was carried out. The compound apparently

was the meso form of diethyl tartarate. Although the first mentioned compound was not identified there is reason to suppose that glyoxylic acid constituted the major portion of the remainder of the oxidation products. This would account for the remarkable naphthoresorcinol test obtained at various points in the treatment of the reaction mixture. Bergmann and Wolff (39) oxidized ∝ methyl glucoside with bromine in the presence of barium hydroxide and obtained an excellent yield of glyoxylic acid which they describe as giving a beautiful color with naphthoresorcinol reagent.

Conclusions from these and other similar experiments were that iodine is an unsuitable oxidizing agent for the production of glucuronic acid, from methyl glucoside. Iodine in conjunction with potassium iodide and potassium hydroxide was also used, and while the naphthoresorcinol test always provided evidence that glucuronic acid was present, none could be separated out by the usual methods of procedure.

Oxidation by potassium permanganate.

It was thought that potassium permanganate might provide a good oxidizing medium in that its strength is easily standardized, the manganese is easily removed, and the potassium salt of glucuronic acid is easily crystallizable. Accordingly, the following experiment was carried out. 5 grams of ∝ methyl glucoside were dissolved in 50 c.c. of water. To this solution was added .9 c.c. 18 N sulphuric acid and 35 c.c. of potassium permanganate solution (1.62 gr./c.c.) and the mixture allowed to stand at room temperature. A precipitate of manganese dioxide settled out after three hours, while after standing for two days, this disappeared and left a clear, colorless solution. Tests with naphthoresorcinol and the Klett colorimeter indicated the presence of 2.24 grams of glucuronic acid. The products of oxidation were not

identified, but glucuronic acid, if present at all, was there only in very small amounts and could not be separated out by the usual methods.

(ii) The effect of Pressure.

Considering the fact that Leutgoeb and Heinrich (40) used eleven atmospheres pressure, in their electrolytic oxidation method, we investigated the effect of pressure on the oxidation of α methyl glucoside by hydrogen peroxide. A high pressure will control the rate of decomposition of hydrogen peroxide according to the reaction

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

which allows the use of a smaller excess of hydrogen peroxide than that which was found to be the most advantageous under atmospheric pressure. It was thought too, that pressure might prevent the splitting of the glucose molecule which has been shown to take place by Jackson and Hudson (36). Two oxidation mixtures were prepared containing 5 grams of methyl glucoside, 80 c.c. of water and 5 c.c. of ferric chloride. 3 c.c. of 30% hydrogen peroxide were added at the beginning, 2 c.c. more after 17 hours and 2 c.c. more after 43 hours. The mixtures were kept at 50°C for a total period of 132 hours. One solution had a capillary tube sealed into the stopper to keep the pressure at atmospheric without allowing evaporation and the other flask was put under pressure, using a car pump and a tire valve sealed into the stopper. Results can be summarized as follows,

	Klett reading	% conversion
Mixture under high pressure	47.8	13.10%
Mixture under medium pressure	43.9	10.78%
Mixture under atmospheric pressure	42.8	10.12%

From these results it was concluded as a result of colorimetric determination, that it is best to oxidize under pressure, although there

is relatively little difference obtained under low and high pressure--at least at the pressures obtained in our mixtures. Moreover, the color of the oxidation mixture, when kept under pressure, remained lighter, showing less breakdown of the glucose molecule.

(iii) The effect of temperature.

The effect of temperature has been investigated. Increase of temperature would be expected to speed up the various reactions going on simultaneously in any mixture which was used. The question was whether or not an increase of temperature would favor the reaction involving hydrogen peroxide and the primary alcohol group. It was noted that higher temperatures caused the reactions involved to proceed faster, as noted by the decomposition of the hydrogen peroxide. At elevated temperatures, the mixture turned a dark color indicating deep-seated decomposition of the methyl glucoside molecule. Aqueous solutions of glucuronic acid are colorless. It was decided that it is best to carry out the oxidation reaction at a temperature in the neighbourhood of 50°C, when hydrogen peroxide is used as the oxidizing agent.

(iv) Effect of P^H

Experiments were carried out to determine P^H changes during the course of the oxidation. Five solutions were prepared each containing 1.94 grams of α methyl glucoside, 10 c.c. of water, 10 c.c. copper acetate solution (.0001 grams/c.c.) and 3.7 c.c. of 30% hydrogen peroxide. The P^H was adjusted to the values listed below with .1 N sodium hydroxide. Oxidation was carried out at 40°C for 12 hours in sealed pressure flasks.

TABLE III.

Solution	P^H adjusted to	P^H after oxidation
1	6.15	2.22
2	8.6	2.4
3	6.98	2.22
4	9.5	2.22
5	7.3	2.31

Change of P^H over a period of oxidation was next investigated. The results are summarized below.

TABLE IV.

Solution	0 Hrs.	16½ Hrs.	42½ Hrs.	95 Hrs.
1) 10 c.c. $Cu(AC)_2$ solution 10 c.c. H_2O 1.5 c.c. H_2O_2	6.52	6.58	6.72	
2) 20 c.c. H_2O 1.5 c.c. H_2O_2	8.82	8.46	8.15	8.18
3) .97 Gr. ∝ methyl glucoside 10 c.c. $Cu(AC)_2$ solution 10 c.c. H_2O 1.5 c.c. H_2O_2	6.82	4.72	3.82	
4) .9 gr. ∝ methyl glucoside 10 c.c. H_2O 10 c.c. $Cu(AC)_2$ solution	7.05	3.75	3.28	3.1
5) 2 c.c. CH_3OH 10 c.c. H_2O 10 c.c. $Cu(AC)_2$ solution 1.5 c.c. H_2O_2	6.66	5.50	5.12	4.95

From the P^H changes noted, it is evident that oxidation is accompanied by the production of some acid. It is to be noted that glucose and methyl glucoside give nearly identical results. This may be due to the fact that

production of acid causes hydrolysis of the methyl glucoside with subsequent oxidation of glucose in the solution originally containing methyl glucoside.

Experiments were conducted to determine the relationship between the P^H and the amount of glucuronic acid produced as indicated by the naphtho-resorcinol test. Five solutions containing one gram of α methyl glucoside and 2 c.c. of 30% hydrogen peroxide were prepared and mixed with the additional reagents listed below. The P^H before oxidation was adjusted with .15 N sodium hydroxide.

TABLE V.

Solution	P^H before oxidation	P^H after oxidation	Color of solution	colorimetric reading Dil. of 1/100
1) 20 c.c. H_2O	7.2	2.78	dark amber	48.8
2) 10c.c. H_2O 10c.c. $Cu(AC)_2$	6.78	2.82	light amber dark residue	82.1
3) 15 c.c. H_2O 5 c.c. $Cu(AC)_2$	6.80	2.80	pale yellow white residue	57.3
4) 10 c.c. H_2O 10 c.c. $Fe(OH)_3$	6.80	2.78	clear red	29.0
5) 15 c.c. H_2O 10 c.c. $Fe(OH)_3$	6.85	2.75	dirty brown plus residue	24.0

From these results it was concluded that there is no correlation between the final P^H of the solution and the amount of oxidation as shown by the Tollen's test.

The nature of the acid produced was investigated in one of the above reaction mixtures. Number (3) was vacuum distilled. The distillate had a

P^H of 2.98, reduced hot silver nitrate solution and gave a red ring test with resorcinol. Therefore, at least some of the acid produced is volatile and has reducing properties. It is significant to note that the distillate had practically the same P^H as the original oxidation mixture at the end of the oxidation period.

The following conclusions were reached with regard to P^H changes in oxidation mixtures of methyl glucoside and hydrogen peroxide with ferric chloride as a catalyst. Temperatures employed were in the neighbourhood of 50°C and pressures were those developed in sealed pressure flasks.

1) Irrespective of to what value the P^H was adjusted at the beginning of the oxidation period, the final value always lies between 2.2 and 2.8.

2) Oxidation may result in the production of an acid strong enough to hydrolyze methyl glucoside into its two components. Subsequent oxidation results in the splitting of the glucose molecule to produce acids of two, three or four carbon atoms.

4) The acid consists of at least two fractions, first a non-volatile fraction and secondly a volatile fraction which has reducing properties. Formic acid is suggested as the most likely. The larger portion of the acid produced is volatile. For example, in one experiment the results were as follows.

Methyl glucoside used=.0258 equivalents

Total acid produced - .0331 equivalents

Volatile fraction of acids - .0214 equivalents

Non-volatile fraction of acids - .0117 equivalents

(v) Catalysts and their effects.

It was hoped that some catalyst could be found which would be specific

for the oxidation of the primary alcohol group; associated with the sixth carbon atom of the methyl glucoside molecule.

Various workers have investigated the use of catalysts in the oxidation of carbohydrates. Craik (38) found that maltose was not affected by hydrogen peroxide except in the presence of ferrous sulphate. Smolenski (35) claimed to have oxidized ∝ methyl glucoside using ferric hydroxide as a catalyst. Copper acetate has been used in the oxidation of carbohydrates. It was decided therefore, to investigate a number of metallic ions with regard to their catalytic properties in the presence of hydrogen peroxide.

The catalyst as designated below is based on mole equivalents of metallic ions in most suitable ion concentration as found by Gwyn (54).

1 7.4×10^{-6} moles

2 14.8×10^{-6} moles

3 29.6×10^{-6} moles

2 c.c. of 30% hydrogen peroxide, .97 grams of ∝ methyl glucoside and 35 c.c. of water made up the oxidation mixture. The flasks containing the mixtures were sealed and left standing at room temperature in the dark for one month. Copper, thallium, selenium, and iron were investigated. Results are as follows.

TABLE VI.

Copper	Klett reading	State of Pressure
c - 1	129	maintained
c - 2	45	no pressure
c - 3	62.5	maintained

TABLE VI(continued)

Thallium	Klett reading	State of Pressure
T - 1	23.5	no pressure
T - 2	25.0	no pressure
T - 3	66.0	maintained
Selenium		
S - 1	13.5	maintained
S - 2	0	none
S - 3	12.0	none
Iron		
F - 1	129.0	maintained
F - 2	45	none
F - 3	62.5	maintained

According to these results, iron and copper seem to be about equal in their catalytic effect. Subsequent work showed that iron was more effective than copper and was therefore used throughout most of the work. In the cases of both copper and iron, the smallest amount of metal proved to be the most effective. In the case of iron 7.4×10^{-6} moles was the most effective concentration. It is to be noted, too, that pressure had considerable effect, the higher pressures giving greater colorimetric readings.

(vi) Specificity of naphthoresorcinol test.

As stated at the outset, the problem was approached from the theoretical point of view. All conditions affecting the oxidation process were considered in some detail. Amounts of glucuronic acid produced were measured

by means of the color produced in ether solution with naphthoresorcinol reagent. Intensities of colors were measured by means of the Klett-Summerson photo-electric colorimeter. The color produced by glucuronic acid and naphthoresorcinol in the presence of concentrated hydrochloric acid has been used to determine glucuronic acid since the test was proposed by Tollens (61). Maughan, Evelyn, and Browne (58) modified this test for the quantitative determination of glucuronic acid and state that there are no interfering substances except mucic acid. More recently Fashena and Stiff (62) have investigated the naphthoresorcinol test for glucuronic acid. Pentoses, as well as glucuronic acid, yield condensation products with naphthoresorcinol which are soluble in ether containing small amounts of alcohol. The addition of 2 c.c. of alcohol to 15 c.c. of ether when making extractions of the colored compound from water, was recommended by Maughan et al (58).

As a result of our inability to separate glucuronic acid in spite of good indications of its presence with naphthoresorcinol, it was decided to investigate the reaction of the reagent with substances which would possibly be produced in our reaction mixtures. Bergmann and Wolff (39) reported that glyoxylic acid gives a strong naphthoresorcinol test. The following substances were tested in amounts which might be present in the dilutions used for testing for glucuronic acid in reaction mixtures. Colors given are those produced in the ether layer.

TABLE VII.

Substance	Color produced
.04 mg. glyoxylic acid	purple
.05 mg. glucose	purple

TABLE VII (Continued)

Substance	Color Produced
.05 mg. methyl glucoside	cloudy purple
.05 mg. gluconic acid	none
.04 mg. tartaric acid	none
.04 mg. saccharic acid	none

In larger quantities, tartaric acid gave a definite color reaction, as shown in the case of diethyl tartarate separated from an oxidation mixture using barium iodide and iodine as the oxidizing agent. A second unidentified fraction separated from the same oxidation mixture also gave a good test with naphthoresorcinol. As a result of these findings, it was doubted whether glucuronic acid was present in the reaction mixtures in which it was indicated by naphthoresorcinol reagent. This conclusion was confirmed by our inability to separate out the acid by the usual methods of procedure.

(vii) Methods of separation.

Various methods of separation suggested themselves. The sodium and potassium salts of glucuronic acid are crystallizable. The barium and calcium salts are amorphous and fairly insoluble in water and more so in alcohol. The brucine and cinchonine salts have been suggested, the first by Smolenski (35) and the second by Leutgoeb and Heinrich (40). A question arose as to whether it was best to form a salt of methyl glucuronide immediately after oxidation or to hydrolyze the methyl glucuronide and then to remove all other constituents except glucuronic acid. Both methods were tried. If present, even in a complex mixture, glucuronic acid should be fairly easily separated. Glucuronic acid itself is slightly soluble in

alcohol. When a water solution of the acid is boiled, evaporated or even allowed to stand at room temperature, water is lost with the formation of the lactone. The anhydride is insoluble in alcohol but readily soluble in water.

In the methods using potassium hydroxide plus potassium iodide and iodine as oxidizing agents the following procedure was followed. The iodine was removed after the expiration of the oxidation time with basic lead carbonate and finally with silver acetate, the precipitates being filtered off. The resulting filtrate was hydrolyzed for three hours with 2 N sulphuric acid, at the end of which time the sulphate ion was removed with barium hydroxide. Excess barium was removed with carbon dioxide. The clear solution was evaporated to small volume to produce a thick syrup. Hot glacial acetic acid was added, the solution allowed to cool and then several volumes of 95% alcohol added. A white precipitate settled out which was not glucurone, as should have been the case if glucuronic acid had been present in the solution. The product was not identified. Similar methods were employed when potassium permanganate was used as the oxidizing agent with similar negative results.

The method employing barium iodide and iodine has been described before. After removal of all metallic ions, a white crystalline substance was precipitated from alcohol which proved to be the meso form of diethyl tartarate. The yield of this substance was high, showing that the major part of the oxidation products was tartaric acid, and that the glucose molecule was split into at least two parts. A second oxidation product believed to be glyoxylic acid was not fully identified.

The brucine salt as recommended by Smolenski (35) was used in an attempt to separate glucuronic acid from the reaction mixture. 10 grams of methyl

glucoside, 19.6 c.c. of 30% hydrogen peroxide and 5 c.c. of ferric chloride solution(.0001 gr./c.c.) were allowed to stand in a pressure flask for ninety-six hours. The resulting solution gave a strong naphthoresorcinol test for glucuronic acid. The mixture was distilled under a high vacuum to remove any volatile acids produced. The distillate had reducing properties. The residue was taken up in water and to this solution was added excess brucine. The solution was refluxed for three hours, during which time there was a darkening in the color of the mixture. After the solution cooled, excess brucine was removed by extraction with chloroform. The final solution supposedly containing the brucine salt of methyl glucuronide gave a good naphthoresorcinol test. This solution was evaporated down to dryness, under a high vacuum and the residue taken up in hot ethyl alcohol. On cooling, fine crystals separated out, which on recrystallization from alcohol gave a sharp melting point of 165°C. The product was identified as ∝ methyl glucoside. Further work on solutions and precipitates failed to extract brucine glucuronide, which if present must have been there in minute quantities. This experiment proved one thing, at least; that ∝ methyl glucoside is stable under the conditions employed. The presence of reducing substances in the distillate indicated some breakdown in the glucose molecule.

CONCLUSIONS AND DISCUSSION.

a) Chemical method.

Early work was undertaken to discover the various influencing factors in the oxidation of ∝ methyl glucoside. Conclusions as to the amount of glucuronic acid present were based on the naphthoresorcinol test. Later, when work was begun on methods of separation, and negative results were

obtained, it was decided to investigate further the specificity of the test. As a result of experiments on this phase of the work, it was decided that previous indications of glucuronic acid were due to interfering substances and this conclusion was strengthened by our repeated inability to separate the acid. Other workers, particularly Smolenski (35) claim to have isolated the substance in yields up to 30%, but our work fails to corroborate these assertions. It is still believed, however that glucuronic acid should be capable of production as a result of the oxidation of the sixth carbon atom of α methyl glucoside. Other reactions may go on concurrently, at greater speeds than the desired one, so that yields of glucuronic acid will necessarily be small. It is believed that other parts of the glucose molecule are more susceptable to oxidation than the primary alcohol group, so that, although this group may be oxidized, the molecule is destroyed with the production of shorter chain acids or aldehydes. The problem resolves itself into finding some specific agent for the oxidation of the primary alcohol group. This specific action might be found in a catalyst and further work could be done on this phase of the chemical investigation.

The biochemical method seems to offer the best method to date of obtaining glucuronic acid. The animals necessary are inexpensive both to obtain and to keep and the yields are good. From dogs a yield of 80% has been obtained. Rabbits can be made to yield up to 50% of the substance.

SUMMARY.

1) The biochemical method for the production of glucuronic acid, using rabbits has been investigated. Modifications in the manner of administering menthol to rabbits have been made. Procedures are given.

2) The available methods of chemical synthesis have been reviewed and discussed.

3) The results of investigations on the chemical production of glucuronic acid from ∝ methyl glucoside are given. The findings indicate that it is not practical to produce the acid by the oxidation of a glucoside or of a compound of glucose which has the aldehyde group alone, inactivated, unless a specific agent is found for the oxidation of the sixth carbon atom.

---------- o o O o o ----------

BIBLIOGRAPHY

1. Quick, A. J., Jour. Biol. Chem. 97, 403, (1932).
2. Fischer, E., Ber. 24-521, (1891).
3. Pryde, J., Williams, R. T., Biochem. J. 30, 794, (1936).
4. Quick, A. J., Jour. Biol. Chem. 70, 379,(1926).
5. Quick, A. J., Jour. Biol. Chem. 98, 537, (1932).
6. Lipschitz, W. L., Bueding, E., Jour. Biol. Chem. 129-333, (1939).
7. Hemingway, A., Pryde, J., Williams, R. T., Biochem. J. 28, 136, (1934).
8. Fishman, W. H., Jour. Biol. Chem. 127, 367, (1939).
9. Cohen, S. L., Marrian, G. F., Biochem. J. 30-57,(1936).
10. Venning, E. M., Browne, J. S. L., Proc. Soc. Exp. Biol. and Med. 34, 792, (1936).
11. Loeb, L., J.Am. Med. Ass'n, 104, 1597, (1935).
12. Lacassagne, A., Compt. Rend., 195-630,(1932).
13. Burrows, H., Brit. Jour. of Surgery, 23, 191, (1935).
14. Ward, H. B.,"Some Fundamental Aspects of the Cancer Problem", P. 51-66 The Science Press, New York,(1937).
15. Boyland, E., Levi, A. A., Biochem. J. 29, 2679,(1935) and Biochem. J. 30, 728,(1936).
16. Willand, H., Dane, E., Ztschr. Physiol. Chem., 219, 240, (1933).
17. Doads, E. C., Goldberg, L., Lawson, W., Robinson, R., Nature 141, 247, (1938).
18. Waters, L. L., Yale J. Biol. Med., 10, 179, (1937).
19. Shear, M. J., Am. J. Cancer, 28, 334, (1936).
20. Ward, H. B., "Some Fundamental Aspects of the Cancer Problem", P. 121-156, Science Press, New York. (1937).
21. Keilin, D., Proc. Roy. Soc. (Lond) B111, 280 (1932).
22. Murphy, V. B., Sturm, E., Jour. Exp. Med., 60, 293, (1934).
23. ibid 60, 305, (1934).
24. Fishman, W. H., Jour. Biol. Chem., 131, 225, (1939).

BIBLIOGRAPHY (Continued).

25. Haddow, A., Robinson, A. M., Proc. Roy. Soc. B 122, 442, (1937).

26. Nitta, Y., Japan J. Obstet. Gynecol., 19, 512, (1936).

27. Quick, A. J., Jour. Biol. Chem., 74-331, (1927).

28. Bang, I., Biochem. Z. xxxii 45, (1911).

29. Williams, R. T., Biochem. J., 34, 272, (1940).

30. Williams, R. T., Biochem. J. 32, 1849, (1938).

31. Nef, J. U., Ann. 357-214, (1907).

32. Killiani, H., Ber. 54B, 456, (1921).

33. Jolles, A. Monatsh., 32, 623.

34. Fischer, E., Piloty, O., Ber. 24, 521, (1890).

35. Smolenski, K., Roczniki. Chemji. 3, 153, (1924).

36. Jackson, E. L., Hudson, C. S., J. Am. Chem. Soc., 59, 994, (1937).

37. Glattfield, J. W. E., Hanke, M. T., J. Am. Chem. Soc., 40-973, (1918).

38. Craik, J., J. Soc. Chem. Ind., 43-171 7T (1924).

39. Bergmann, M., Wolff, W. W., Ber. 56B, 1060 (1923).

40. Leutgoeb, R. A., Heinrich, H., J. Am. Chem. Soc., 61-870 (1939).

41. Stacey, M., J. Chem. Soc., (1939) 1529.

42. Zervos, L., Sessler, P., Ber. 66 B. 1326, (1933).

43. Goebel, W. F., Babers, F. H., Jour. Biol. Chem., 100, 573, (1933).

44. Goebel, W. F., Babers, F. H., Jour. Biol. Chem., 100, 743, (1933).

45. ibid 101, 173, (1933).

46. ibid 106, 63, (1934).

47. ibid 110, 707, (1935).

48. ibid 111, 347, (1935).

49. Hotchkiss, R. D., Goebel, W. F., Jour. Biol. Chem., 115, 285, (1936).

50. Pryde, J., Williams, R. T., Biochem. J. 27, 1205, (1933).

BIBLIOGRAPHY (Continued).

51. Hawk, P. B., Bergeim, O., "Practical Physiological Chemistry", 11th Ed. P. 437, Blakeston & Son. (1937).

52. Jacobi, H., Ann. 272, 170, (1893).

53. Wohl, A., Ber. 26-730, (1893).

54. Gwyn, A., Bachelors Thesis 1940, The University of British Columbia.

55. Patterson, T. S., Robertson, J., J. Chem. Soc. 300, (1929).

56. Koenigs, W., Knorr, E., Ber., 34, 959, (1901).

57. Fischer, E., Raske, K., Ber. 42, 1465, (1909).

58. Maughan, G. B., Evelyn, K. A., Brown, J.S.L., Jour. Biol. Chem., 126, 567, (1937).

59. Mozolowski, W., Biochem. J., 34, 823, (1940).

60. Goebel, W. F., Jour. Biol, Chem., 72, 801, (1927).

61. Tollens, B., Z. Physiol. Chem., 61, 95, (1909).

62. Fashena, G. J., Stiff, H. A., Jour. Biol. Chem., 137, 21, (1941).

---------- o o O o o ----------