The Composition and Toxic Effects of Gossypol

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GOSSYPOL is a peculiar phenolic body which, as far as is known, occurs only in the cotton plant. The substance possesses many characteristic chemical properties that give it a unique academic interest. From the practical standpoint, also, the substance is important, as its toxic properties are conceded to be responsible for the so-called cottonseed injury which results from the feeding of large quantities of cottonseed meal to livestock (1). This injury constitutes a problem of considerable economic magnitude, the solution of which will eventually involve a method for detoxifying cottonseed meal. In the past an intelligent approach to such an endeavor has been greatly hindered by the lack of specific information regarding the chemistry of gossypol. In order to overcome this difficulty, if possible, a new chemical study of the substance was undertaken a little over a year ago. The purpose of this report is to give an outline of the results of this work. Before presenting the report, however, a summary of the chemical work previously done upon gossypol will be given in order to acquaint the reader with the status of the problem at the time this investigation was undertaken.

Undoubtedly gossypol was first prepared in a fairly pure condition by Marchlewski (2) in 1899. His preparation contained loosely bound acetic acid and had a melting point of 188°C. He reported analyses of a number of samples, but he was not able to establish with certainty a molecular formula although $C_{13}H_{14}O_4$ or $C_{32}H_{34}O_{10}$ was suggested. He made no crystalline derivatives of the material but showed that it was not a glucoside and that in all probability it contained no alkoxyl groups. He recorded some color reactions and its behavior toward certain reagents.

Carruth's Investigations

No further outstanding chemical work was published upon the subject until Carruth (3) communicated his interesting results in 1918. This investigator reported improvements in the preparation of gossypol, thus making possible more extended studies. As a result of his investigation he concluded that gossypol is a phenolic substance, having the formula $C_{30}H_{30}O_9$ or $C_{30}H_{28}O_9$. When the material was crystallized from acetic acid it contained one molecule of loosely bound acid which could be removed by dissolving the substance in ether, adding water, and then removing the ether by evaporation. The acetic acid remained in the water and could be titrated. In this way a value near 532 was obtained for its molecular weight. Determinations of this constant by physicochemical methods were not conclusive. Values ranging from 300 to 595 resulted by the use of different methods and solvents. When gossypol was dissolved in

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aniline, or when gossypol and aniline were dissolved in an appropriate solvent, a characteristic crystalline product was formed. Carruth assumed the compound to have the formula $C_{30}H_{28}O_9.2C_6H_5NH_2$. No carbon or hydrogen figures were given on this material, but those for nitrogen were reported to be from 3.97 to 4.84 per cent. Crystalline acyl derivatives of gossypol were not obtained, but analysis of the amorphous acetyl derivative indicated the presence of four acetyl groups. When gossypol was heated to its decomposition point a new substance called *B* gossypol was formed. This was obtained crystalline and apparently represented gossypol less two mols of water.

Further Work Desirable

The work of Marchlewski and of Carruth constitutes essentially all that was recorded concerning the chemistry of this interesting substance at the time the present investigation was undertaken; but a critical study of their findings indicated that reexamination of the data given was desirable. A quantity of gossypol was therefore prepared and purified (4). The results obtained upon this material indicated that the molecular formula for gossypol is $C_{30}H_{30}O_8$. This formula differs from Carruth's, but, in the light of the experience gained in the purification of the substance, it seems doubtful whether his material was entirely pure. A reddish brown impurity was associated with all the gossypol prepared by Carruth's method that was removed only with great difficulty by a large number of solvents suitable for its recrystallization. A combination of solvents that gave the desired result was eventually found, but again difficulty was encountered in obtaining the material free from the solvent used in its purification. In a number of cases, a small indefinite amount of solvent remained with the crystals. This could not be removed in a 0.1 mm. vacuum over both sulfuric acid and potassium hydroxide at temperatures which would insure the integrity of the substance. Several solvents were found, however, which under certain conditions gave material having uniform composition. The method used to verify this was to obtain from different solvents preparations that gave check analyses. When this was accomplished, carbon and hydrogen figures were obtained which indicated the formula given above. Various derivatives, prepared from the purified gossypol, also gave values upon analysis which checked the formula indicated. Among these were the so-called "acetate," i.e., gossypol + one molecule of acetic acid, anhydrogossypol (Carruth's B gossypol), the aniline condensation product, the dioxime, and a hexaacetyl derivative.

Molecular Weight Studies

The analytically pure gossypol was a bright canary-yellow crystalline substance with a melting point of 214° (corrected). In solution no optical activity was exhibited. Molecular weight determinations by the camphor method of Rast (5) gave results of the order required for the formula suggested. Also, the acetic acid in the so-called acetate was found to be 10.51 per cent, which gave a molecular weight of 511 for gossypol, if it is assumed that one mol ot the latter is associated with one mol of acetic acid. Gossypol was soluble in ether and acetone, but only sparingly soluble in other common organic solvents. It was insoluble in water, but dissolved readily in dilute ammonia and sodium carbonate. It was soluble in aqueous fixed alkalies; but when dissolved in an excess of alkali it slowly decomposed, forming a brown solution which gradually became a beautiful purple. However, when potassium hydroxide was added to an aqueous suspension of finely ground gossypol, in amounts slightly more than enough to cause complete solution, the excess alkali could be titrated and the acid value of the substance was thus determined. If it is assumed that two equivalents of potassium hydroxide were used, molecular weight values of 505 and 507 were obtained. Gossypol dissolved in concentrated sulfuric acid formed a deep scarlet colored solution.

Anhydrogossypol

Upon diluting this acid liquid with water, the gossypol was thrown out unchanged. It was readily soluble in saturated methyl alcoholic hydrochloric acid. At first the color of the solution was brown, which gradually turned deep green and finally intense blue. When heated to its melting point, gossypol lost two mols of water, producing a new crystalline body, anhydrogossypol, $C_{30}H_{26}O_6$. This material possessed markedly different solubilities, but still retained the characteristic color reactions of the original material. Oximation of gossypol produced a colorless dioxime, which seemed to indicate that the chromophores are quinone groups. As stated by Carruth, gossypol combined with aniline to form a deep orange-colored crystalline compound. This substance was found to be a condensation product formed by one mol of gossypol uniting with two mols of aniline, with the elimination of two mols of water. Its formula was found to be $C_{42}H_{40}N_2O_6$, and it is probably a Schiff base type of compound.

Results of Acetylation

Acetylation of gossypol was carried out with acetic anhydride, with the use of sodium acetate and also pyridine as catalysts. With sodium acetate, by employing the conventional proportions of reagents (one part of acetate, four parts of substance, and sixteen parts of acetic anhydride) and by boiling the mixture for ten minutes, a non-crystallizable product was obtained which analyzed for 30.8 per cent acetyl. But when a large excess of the reagents was used and the boiling was continued for two hours, a material resulted, about ten per cent of which was obtained as beautiful crystalline plates. Acetylation in pyridine solution gave the same product but the yield was larger. This substance, when analyzed for acetyl by Perkins' method (6) for O-acyl compounds, gave from 24.9 to 25.9 per cent acetyl. These figures agreed well for a tetraacetyl gossypol (25.07 per cent). Carbon values, however, were those of a hexaacetyl body. It was then found that if Perkins' method (6) for N-acyl compounds was used, the percentage of acetyl required for a hexaacetyl derivative was obtained. The crystalline material was therefore a hexaacetyl gossypol, indicating that in the parent substance there were six hydroxyl groups. Thus all the oxygen in gossypol has been accounted for. Two atoms are present as carbonyl groups, as was shown by the formation of a dioxime, and six are present as hydroxyl groups. It is to be emphasized, though, that two of these hydroxyl groups behave differently from the remaining four. This was manifest by the drastic treatment necessary to remove the last two acetyl groups, and also by the fact that two hydroxyl groups are capable of being titrated as an acid.

In the course of the purification of the substance it seemed desirable to determine the approximate toxicity of the analytically pure gossypol as well as some of the less pure preparations being dealt with. The results are recorded in Table I. In this work certain observations were made which have not hitherto been recorded, but which seemed to be of sufficient interest to warrant reporting.

White rats, whose average weight was 190 gm., were injected intraperitoneally with cottonseed oil solutions of the various preparations.

There was no apparent difference between the toxicity of the crude gossypol acetate and the analytically pure gossypol. Rats receiving doses of twenty mg. or more per kilo of body weight died in from twenty hours to thirteen days. Of those receiving from ten to twenty mg. per kilo, fifty per cent died in from two to eighteen days; the remaining fifty per cent survived. Though the percentage of deaths at fifteen mg. per kilo equals that at twenty mg. per kilo, death resulting from the latter dosage was the result of acute poisoning, whereas death at fifteen mg. per kilo was the result of chronic poisoning.

Postmortem findings in the acute type of poisoning were oily droplets and plaques in the peritoneum, congested lungs, hemorrhagic condition of all the viscera, and cardiac dilatation. In the chronic type of poisoning the outstanding indication was intestinal impaction. The cause of delayed death was stated by Dr. Formad, pathologist of the Bureau of Animal Industry, "to be probably due to a paralyzing effect upon the sympathetic nervous system." Dr. Formad further stated that "it seemed probable that there was a tonic spasm of the sphincter ani accompanied by general decreased intes-

TABLE I

Toxicity Tests Made Upon Different Preparations of Gossypol

The material, dissolved in cottonseed oil, was given intraperitoneally to white rats, the average weight of each being 190 gm.

Material Used	Dose, Mg. Per Kg.	No. of Rats Used	No. of Deaths Resulting	Time in Days Until Death	Remarks
Crude gossypol acetate	25	1	0	0	
	50	2	2	2, 2	Acute
	100	1	1	1	Acute
Gossypol recrystallized from chloroform	25	2	2	6, 13	
	50	2	2	2, 2	All but 13 day animal acute
	75	2	2	1, 1	
	100	2	2	1, 1	
Gossypol twice recrystallized from ether and petroleum ether	20	2	2	7, 10	
	30	2	2	3, 5	All acute type
	40	1	1	3	
	50	1	1	1	
Purified gossypol recrystallized from ether (analytically pure)	10	6	2	17, 18	2 day and 3 day animals acute, all others, chronic
	15	6	5	10, 11, 12, 12, 13	
	20	6	5	2, 3, 9, 10, 10	

tinal tonus which led to decreased rate of elimination of feces and finally to an intestinal impaction. This latter, in turn, produced pressure upon the diaphragm which was transmitted to the heart and lungs and served as a probable cause of death."

Condition of Gossypol in Cottonseed Meal

The molecular formula of gossypol and its approximate toxicity having been determined, attention was next directed to the condition of the bound gossypol in cottonseed meal (7). As is well known among those interested in the cottonseed meal problem, the cooking and pressing processes to which cottonseeds are subjected in the manufacture of cottonseed oil exert a profound change in the condition of the gossypol originally in the raw kernels. Gossypol is readily extracted from the raw seeds with solvents such as ether or acetone. When cottonseed meal is treated in the same manner, however, the quantity of gossypol obtained is much less than that extracted from the raw kernels, although little or none of the substance is present in the expelled oil (8). Coincident with the diminution of extractable gossypol, the toxicity of the meal as compared with that of the untreated seeds becomes much less (3, 8, 9, 10). Some investigators have explained this phenomenon as being due to a change in the gossypol molecule that is supposed to occur during the cooking and pressing process. Withers and Carruth (8, 9, 10) have advanced the idea that the reaction is mainly one of oxidation. The oxidized gossypol, which is considered to be non-toxic, or at least much less toxic than the parent material, has been called by Carruth (9) D gossypol. More recently Sherwood (12) has suggested that the transformation of gossypol to D gossypol is one of hydrolysis. In each case the assumption was made that D gossypol is an individual substance derived from gossypol through an alteration of the molecule of the latter. The support of this idea, however, rests upon indirect evidence only, as D gossypol has never been isolated. On the other hand, hot aniline extraction of cottonseed meal gives a crystalline material having the general appearance of dianiline gossypol. The substance has been considered a derivative of D gossypol and in the literature it is known as aniline D gossypol; but as no thorough analysis of it has been recorded, it has been impossible to arrive at any definite conclusions concerning its nature.

Condensation Products with Aniline

In connection with this phase of the gossypol problem, aniline D gossypol was investigated and found to be identical with dianiline gossypol which is obtained by condensing pure gossypol with aniline. The evidence upon which this statement is made is: The two substances have the same melting point, and no depression occurs when they are mixed. They possess the same optical properties. Their molecular weights are the same within the limits of experimental error. Their elemental composition is identical, and finally, upon hydrolysis of aniline D gossypol, a substance is obtained which is identical both chemically and physiologically with analytically pure gossypol. This evidence indicates that the gossypol molecule has undergone no chemical alteration, such as oxidation or hydrolysis as suggested by the investigators mentioned before.

Bound Gossypol Hypothesis

Furthermore, the information materially simplifies the problem of the fate of gossypol during the cooking and pressing treatment and leads to a hypothesis concerning the mechanism of the process whereby gossypol becomes bound and is also rendered more or less physiologically inert. This hypothesis is as follows: During the heating and pressing to which the seeds are subjected the resin glands containing the gossypol are disrupted and possibly much of the gossypol is dissolved in the oil present. It thus comes in intimate contact with the proteins of the seeds and in this condition, favored by heat and pressure, probably condenses with free amino groups of the protein molecules, as it does with many primary amines, forming substances *similar in type* to the dianiline gossypol. The material is thus bound rendering it insoluble and as far as is known physiologically inert.* The work of Jones and Waterman (11), in which they found that peptic and tryptic digestion of casein and cottonseed globulin was reduced fifteen per cent when treated with one per cent gossypol, lends support to this idea.

Whether or not this explanation is correct, it can be stated definitely that the cooking and pressing processes used in the manufacture of cottonseed oil produce no chemical alteration in the gossypol molecule. In the light of this information it would seem desirable to discontinue the expression D gossypol, because of its generally accepted meaning, and substitute for it the term *bound* gossypol.

(To be continued)

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*Withers and Carruth (10) advanced a somewhat similar view, suggesting that while most of the gossypol disappears by means of an oxidation process, some may become bound through condensing with amino and carboxyl groups of protein molecules. However, in Carruth's article upon the chemistry of gossypol (3) a statement is made which seems to indicate that he and Withers had subsequently abandoned this hypothesis.

Refining Committee to Have Research Chemist

The Governing Committee of the American Oil Chemists' Society has placed a small sum of money at the immediate disposal of the Refining Committee to be paid as salary to a research chemist for further standardizing refining methods.

This announcement was made recently by Mr. A. W. Putland, president of the American Oil Chemists' Society.