[CONTRIBUTION FROM THE CHEMICAL DIVISION OF THE NORTH CAROLINA EXPERIMENT STATION.]

CONTRIBUTION TO THE CHEMISTRY OF GOSSYPOL, THE TOXIC PRINCIPLE OF COTTONSEED.

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Gossypol is a peculiar coloring matter present in the cotton plant. Apparently it does not belong to any known class of plant pigments. Its relation to cottonseed poisoning has been shown by Withers and Carruth[1] in separate publications on the subject. At present gossypol is of negative economic value, but it is possible that it may prove useful in the future.

Historical.

The first published work on this substance seems to be by F. Kuhlmann.[2] Kuhlmann was attempting to recover fatty acids from the "degras" or "foots" of cottonseed-oil purification. After distilling off most of the fatty acids by superheated steam, a greenish blue mass was left behind, from which he obtained a substance later called "cottonseed blue" by dissolving out the fatty acids with naphtha, to which he gave the formula $C_{17}H_{24}O_4$. This substance prepared by Kuhlmann differs considerably from pure gossypol in composition and properties and perhaps consisted of a mixture of gossypol with its oxidation products and fatty acids.

A substance known as "gossypin" has been prepared from the foots of cottonseed oil. This substance is described[3] as a light brown pungent powder and evidently consisted of gossypol with certain odorous impurities. Probably this description is based on the work of J. Longmore[4] who, however, does not give his substance any name.

Under the title of "Cottonseed Oil, Its Coloring Matter and Mucilage," J. Longmore describes the preparation of the coloring matter from crude cotton oil:

> "Crude cottonseed oil is of an intense ruby, nearly black color due to its holding in solution a powerful vegetable coloring principle. This latter, according to the results obtained by the author, amounts to 10 to 15 pounds per ton of oil... .
>
> This coloring matter has now been isolated by the author with the result of throwing some light on its nature. In the dry state it is a light powder of pungent odor, of a brown earthy color and possesses powerful dyeing properties. A small proportion of it is soluble in water, but the principle bulk dissolves only in alcohol or alkalies. It is quite insoluble in acids."

Longmore employed strong alkalies to salt out the soap in the foots and to keep the gossypol in solution. He isolated the gossypol by adding acid

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[1] *J. Agr. Research*, **5**, 261 (1915); *J. Biol. Chem.*, **32**, 245 (1917); *J. Agr. Research*, **12**, 83 (1918), and a fourth paper to appear soon.

[2] *Compt. rend.*, **53**, 444 (1861).

[3] Cf. Julius Wiesner, "Die Rohstoffe des Pflanzenreiches," 1903, 2nd edition, p. 754.

[4] *J. Soc. Chem. Ind.*, **5**, 200–6 (1886).

to the alkaline solution. He mentions no further purification and it is evident that his product was very impure.

Finally Marchlewski[1] isolated a crystalline product from the "foots" of cottonseed oil by means of a tedious purification process. He named the crystalline substance gossypol from gossyp (ium-phen)ol. His method of preparation is given in the following translation:

> "The crude product which was obtained from an English oil manufacturer had already undergone a purification process consisting of the removal of the greater part of accompanying fatty acids in the form of their calcium salts.
>
> It was extracted with ether which dissolved the gossypol, its oxidation products (which give the solution a dark brown, almost black color) and also considerable amounts of impurities. This solution is concentrated. There remains a viscous mass which is added in portions to boiling acetic acid. When solution takes place it is filtered from a small residue and allowed to cool. After some standing a dark brown amorphous mass separates which is again dissolved in acetic acid. After 4 or 5 repetitions one finally obtains microscopic crystals which are dark brown in color, and which represent the still very impure gossypol.
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> Further purification is best secured as follows: Dissolve the crystalline mass in boiling alcohol and add to the dark brown solution 50% acetic acid until clouding is noticeable. Then the solution is heated to boiling and filtered. After some standing crystals appear which are considerably lighter than the first product. Repeating this process 5 or 6 times finally yields a beautifully crystallized yellow product. Attempts to obtain the gossypol entirely colorless failed. It seems then that the yellow color is inherent although this is not considered as established."

As will be shown later, Marchlewski's "gossypol" was, no doubt, a substance containing one molecule of acetic acid in loose combination with the phenolic body. This compound with acetic acid will be called for convenience gossypol "acetate," and the phenolic body itself *gossypol.*

In a personal communication to the writer, A. G. Perkin states that he had a very large quantity of it in paste form and that Professors Hummel and Knecht carried on experiments in the hope of converting it into a dye of commercial importance but without success. Perkin intended to work with gossypol but refrained when Marchlewski stated that he wished to reserve the subject.

Occurrence.

Gossypol appears to be a constituent of the cotton plant only. It occurs in peculiar glands called "gland dots," "secretion cavities," or "resin glands" which are present in all parts of the plant except the woody tissue. These are 100 to 400μ in diameter and are readily visible to the eye. They appear to be of lysigenous origin, *i. e.*, formed by disintegration of adjacent cells.[2] The author obtained a crude material from an ether extract of the bark which evidently was chiefly gossypol. It was not obtained crystal-

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[1] *J. prakt. Chem.*, 60, 84–90 (1899).

[2] A photomicroscopic study of the formation of these glands has been made by Dr. A. Viehoever and Mr. E. E. Stanford of the Pharmacognosy Laboratory of the Bureau of Chemistry. It is not yet published.

line. The glands in the root-bark, leaf, petals and boll as well as those of the seed give with concentrated sulfuric acid a characteristic red color from which fact it is inferred that gossypol exists in all these. An excellent description of the cross section of the seed, showing situation of the glands is given in Winton's "Microscopy of Technical Products," p. 365.

A. G. Perkin[1] states that gossypol "is not identical with and does not appear to be allied with the coloring matter of the (cotton) flowers."

Amount Present in Cottonseed.

Analyses of parts of the plant other than the seed have not been made. By two different methods, the amounts of gossypol present in the undried kernels, nearly free from lint and hulls, has been found to be about 0.6%. The highest yield recorded was 0.63%.

Attempts have not been made to study the actual variation in different varieties of the cotton plant, but since all varieties of seed seem to have approximately the same number of glands, it would appear that the gossypol content does not vary to a greater extent than the oil or protein content. Assuming for convenience that 100 g. of fresh raw kernels contain 0.6% gossypol and 33.3% oil, then the ether extract of this material would contain about three times 0.6 or 1.8% gossypol. Since extraction by petroleum ether does not remove gossypol, the residue after extraction by petroleum ether or gasoline would contain about 0.9% gossypol.

Preparation.

The methods which have been used to isolate gossypol from cottonseed are much simpler than the tedious process by which Marchlewski secured it from the "foots" of cottonseed-oil purification. A crystalline product of high purity is secured directly.

Method 1.—It has been found most convenient to use decorticated cottonseed kernels or "meats" which have been passed through rollers to crush the kernels before they are cooked in the oil mill. The kernels are flattened out and in this condition are easily broken up by sifting into smaller particles. The material is passed through a coarse sieve to remove the greater part of the lint and hulls, and then is extracted with petroleum ether or gasoline. For preparation of considerable amounts of gossypol the kernels may be simply percolated with gasoline to remove the greater part of the oil. The resulting material is dried and extracted with ethyl ether. The extract amounts to 2 to 6% of the weight of material extracted according to the thoroughness of previous extraction. The concentrated extract is of a dark cherry-red color if fresh kernels have been used or almost black if from older seeds. These "gossypol extracts" contain about 17% to 50% gossypol. In one case where very fresh kernels were used a considerable part of the gossypol separated out of the "gossypol extract"

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[1] *J. Chem. Soc.*, **75**, 825 (1899).

on standing as a red crystalline powder. The gossypol may be precipitated in brown amorphous flocks by adding petroleum ether, but better yields, practically quantitative, are secured by treating the gossypol extract with $^1/_2$ to $^1/_3$ its volume of glacial acetic acid. On standing several hours or days, or more quickly by warming in the water bath, most of the gossypol separates as the well crystallized gossypol "acetate" containing 10.1% acetic acid of composition. The crystalline paste is then sucked off and washed with small amounts of glacial acetic acid, and then with petroleum ether. For further purification the gossypol acetate thus secured is dissolved in ether and glacial acetic acid—about 10 parts to 1 part of gossypol—is added. The ether is then in part distilled until the gossypol begins to separate readily. By this method the fatty and colored impurities are retained by the solvent, which is not so much the case when Marchlewski's method using alcohol and aqueous acetic acid is followed. The crystalline product in this case contains uniformly 10.1% acetic acid while by Marchlewski's method it tended to vary from 8.5 to 9.6%.

For the preparation of crystalline gossypol from the "acetate" the latter is dissolved in ether and water is added. The ether is distilled leaving the gossypol as crusts floating on the water which contains all the acetic acid. The free gossypol may then be crystallized from alcohol or other suitable solvent.

Method 1a.—In this method the kernels are not previously extracted with petroleum ether, but with ethyl ether only. The evaporated ether extract is treated with acetic acid and allowed to stand until the gossypol "acetate" separates out. This requires a much longer time than in Method 1. The use of 80% and 90% acetic acid is more advantageous than the use of the glacial acid. It was found that 7 parts of 80% acetic acid per 100 of extract gave larger yields than did 90% or 100% acetic acid. Acid weaker than 80% tends to form a separate layer with the extract. By adding 7 parts per 100, heating to 100° to 115° and then setting aside, the separation of the gossypol "acetate" is hastened. Nevertheless, this is very slowly formed, requiring one to three weeks. The yields are poorer on account of the large mass of oil present but from 50 to 80% of the gossypol may be obtained if sufficient time is given. The following table summarizes some data obtained with different dilutions of acetic acid:

TABLE I.

7 parts of acetic acid per 100 parts by volume of ether extract. Time of standing, about 2 weeks. Weights of extracts, about 91 g.

Strength of acid. Per cent.	G. Gossypol "acetate." Heated to 100° before setting aside.	Heated to 115°.
99.5	0.0742	0.3040
90	0.6286	0.7170
80	1.1996	0.9310

Method 2.—The basis of this method is the insolubility in oil of the salts of gossypol. This principle is applied commercially in removing the gossypol and other coloring matters from crude cottonseed oil. The extracts (cf. Method 1) of cottonseed kernels are shaken with a slight excess of strong aqueous caustic soda. The sodium salt of gossypol and sodium salts of the free fatty acids are formed and pass into the aqueous layer, while the clear yellow oil rises to the top. The alkaline liquor is neutralized with acid which causes the fatty acids and gossypol to separate as a pasty mass. This mass may be treated with hot acetic acid to cause separation of the gossypol. This method is not satisfactory and is almost useless where much free fatty acid is present in the oil, when a "break" into oily and aqueous layers is greatly retarded. Gossypol quickly oxidizes in alkaline solutions exposed to air. This oxidation, however, may be retarded by adding sodium hyposulfite ("hydrosulfite") $Na_2S_2O_4$.

If little free fatty acid is present in the gossypol extract, the amount of gossypol present may be approximated by the use of standard dilute alkali. Ether and other fat solvents are added, if necessary, to destroy emulsions. Phenolphthalein is used as indicator. The color change occurs in the aqueous layer after the formation of the disodium salt of gossypol.

Method 3.—This method involves the use of aniline as a precipitating agent for gossypol. A very slightly soluble compound,[1] apparently the dianiline salt of gossypol, is formed, which separates out of an oily extract on standing as an orange-yellow micro-crystalline precipitate. Aniline (about 5% of the weight of the extract) is added to an ether extract of cottonseed. The mixture is warmed on the water bath and set aside to stand a week or more. If given sufficient time the yield is practically quantitative and the method has been used to estimate[2] the amount of gossypol in extracts of cottonseed products.

The aniline-gossypol compound is filtered out and washed free from oil, etc., with ether, or with a small amount of ether followed by larger amounts of petroleum ether. It may be purified by recrystallization from aniline.

To prepare gossypol from the aniline compound it is dissolved in hot alcoholic potash to decompose it and the aniline is steam-distilled out. The resultant aqueous solution of potassium "gossypolate" is treated with a pinch of sodium hyposulfite ($Na_2S_2O_4$) in order to reduce any of the blue oxidation product which may be formed. The gossypol is then precipitated in amorphous flocks by acidifying. It may be filtered out or removed from the solution by shaking with ether in a separatory funnel. It may be purified according to Method 1.

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[1] This compound was discovered by Dr. J. T. Dobbins working in this laboratory in attempts to prepare "B" gossypol.

[2] This method therefore may be used to approximate the relative toxicity of extracts. (See *J. Biol. Chem.*, 32, 87 (1917).)

Marchlewski's "Gossypol"—A Molecular Compound of Gossypol and Acetic Acid.

One would not ordinarily expect a substance to crystallize with acetic acid from a mixture of alcohol and 50% acetic acid in which the acetic acid content varied from 20% to 35%. The crystalline product obtained in this way was usually washed with hot water and dried at 100°. Even on heating to 130° or well above the boiling point of acetic acid there was but very slight loss in weight. Later it was established that this substance, gossypol "acetate," does not lose the acetic acid by boiling in water nor in fact readily below temperatures of 180°, at which point the substance decomposes.

This fact was not recognized at first and was not suspected until a series of molecular-weight determinations by the Beckman method with various solvents gave anomalous results. The presence of acetic acid was established by heating the substance in a tube to its melting point or rather to its decomposition point. A black melt was left behind and a condensate having the characteristic odor of acetic acid collected on the tube. The amount of acetic acid present in these earlier preparations was determined by collecting the vapors driven off at 180–200°, or more easily by the method described under determination of the molecular weight by titration of the acid. For the reason that the amount of acetic acid in preparations obtained from alcohol and 50% acetic acid varied slightly (8.5 to 9.5%) and was less than in preparations crystallized from ether and glacial acetic acid, this latter mixture was used as a solvent, yielding a product freer from foreign coloring matter and with a uniform acid content of 10.1%.

Molecular Weight of Gossypol.

It was first sought to determine the molecular weight of gossypol by physical methods. Preparations of gossypol recrystallized from alcohol with the addition of 50% acetic acid were first used. The results with various solvents were not uniform. Thus, while the boiling-point method with ether and acetone indicated a molecular weight of 300 to 350; with alcohol, benzene and carbon tetrachloride, the elevation of the boiling point was abnormally small indicating a much greater molecular weight. The freezing-point method with benzene was unreliable, owing to the slight solubility of the substance in cold benzene. These results led to suspecting the presence of solvent of crystallization. This was found to be the case as previously described. Further molecular-weight determinations with the McCoy apparatus of the gossypol freed from acetic acid gave the following results:

(1) Ether as solvent. Gossypol 1.002 g. Ether 17.73 g. Elevation 0.20°. Molecular weight found: 595.

(2) Acetone as solvent. Gossypol 1.008 g. Acetone 25.6 g. Elevation 0.121°. Molecular weight found: 553.

(3) Gossypol "acetate" in ether as solvent. Gossypol "acetate" 0.994 g. Ether 20.0 g. Elevation 0.291. Apparent molecular weight found: 352.

These data indicate that the gossypol "acetate" dissociates in solution into free gossypol and acetic acid causing the previously mentioned anomalous results.

Molecular Weight of Gossypol by Chemical Methods—Titration with Standard Alkali.—After it was found that gossypol "acetate" crystallized from ether-acetic acid contained uniformly about 10.1% acetic acid or somewhat more than that prepared in Marchlewski's way, the latter's method was abandoned. Since the determination of acetic acid is easily and accurately made, the method offers a reliable method for the determination of the molecular weight.

Gossypol "acetate" recrystallized 4 times from ether-glacial acetic acid mixture was dissolved in ether. Warm water was then added and the ether driven off. The water was then separated from the brittle crusts of gossypol which remained, the latter dissolved in ether, and the previous treatment repeated. The water containing the acetic acid was then titrated with alkali using phenoltetrachlorophthalein as indicator.

Subst., (I) 0.5326, (II) 0.5057; cc. 0.1 *N* NaOH, (I) 8.98, (II) 8.57.
Acetic acid found: (I) 10.12, (II) 10.17.
Acetic acid calc. for:
(1) Mol. wt. 592 $C_{30}H_{28}O_9.C_2H_4O_2$.................................. 10.18
(2) Mol. wt. 594 $C_{30}H_{30}O_9.C_2H_4O_2$.................................. 10.10

Hence "free" gossypol has a molecular weight of 530 if $C_{30}H_{28}O_0$, or 532 if $C_{30}H_{30}O_9$.

Molecular-Weight Determination by Direct Titration of "Free" Gossypol.—The above values for the molecular weight of gossypol stand in agreement with the values obtained by direct titration of the residual gossypol after removing the acetic acid of combination. The free gossypol left after removal of the acetic acid in the above determinations was dissolved in alcohol and titrated with alkali using phenoltetrachlorophthalein as indicator. Since the gossypol solution is yellow the end point is more difficult to detect.

The weights of gossypol used here were found by subtracting the 10.12% and 10.17% acetic acid from the weights of the gossypol "acetate" used above.

Subst., (I) 0.4787, (II) 0.4543; cc. 0.1 *N* NaOH, (I) 17.6, (II) 17.0.

Hence assuming gossypol neutralizes two equivalents of NaOH the values for the molecular weight are (I) 544, (II) 532.

Concordant results were also obtained by titrating weighed amounts of pure gossypol and of gossypol "acetate." The "acetate," as is evident, neutralizes three equivalents of alkali. While gossypol thus behaves as a fairly strong acid, it is not thought that it contains carboxyl groups since the benzoyl and acetyl derivatives are not soluble in cold dilute aqueous alkali. The fact that the disodium salt of gossypol is neutral to phenolphthalein in aqueous solution gives an indication of its relative strength as an organic acid.

Empirical Formula.

Marchlewski[1] made several analyses of gossypol which had been dried under different conditions. Those which had been heated were much higher in carbon. The three determinations on which, together with the analysis of the lead salt, Marchlewski established his formula $C_{13}H_{14}O_4$ (or $C_{32}H_{34}O_{10}$) are given below.

Composition of Gossypol (Marchlewski).

Preparation ash free and dried *in vacuo* over H_2SO_4.

Found: C, 66.5, 66.3, 66.4; H, 6.0, 6.2, 6.1.

Calc. for $C_{13}H_{14}O_4$: C, 66.7; H, 6.0. Calc. for $C_{32}H_{34}O_{10}$: C, 66.4; H, 5.9.

Composition of the lead salt from an alcoholic solution of gossypol and lead acetate. Calc.: C, 35.5; H, 2.7; PbO, 50.8. Found: C, 36.00; H, 3.01; PbO, 51.1.

"Therefore," Marchlewski says, "gossypol contains two hydroxyl groups. Should I succeed in obtaining greater quantities of gossypol in sufficiently pure condition, I will not fail to give this interesting body a thorough study and communicate the results in this place."

Since the following analytical data differ somewhat from those given by Marchlewski, the question arises, are his analyses correct, or did he have a substance which, by reason of the previous treatment of the seed and oil, had undergone a change in composition. Some slight difference is due to the smaller amount of acetic acid probably present in his preparation.

Although evidence shows that considerable alteration in the toxic properties of gossypol takes place in cooking in the oil mill, the changed gossypol ("D" gossypol, see p. 660) does not pass into the oil to any extent, but remains in the press cake, hence it is apparent that Marchlewski did not have "D" gossypol. On the other hand in cold pressing much *unchanged* gossypol passes into the oil.

The following analytical data give results which agree well with the formulas $C_{30}H_{28}O_9$ or $C_{30}H_{30}O_9$ for gossypol.

Analysis of gossypol "acetate" four times recrystallized from ether and glacial acetic acid.

	Subst.	CO_2.	H_2O.	% C.	% H.
	1st crop of crystals.				
1	0.2321	0.5547	0.1197	65.16	5.77
2	0.2263	0.5404	0.1197	65.12	5.92
	2nd crop of crystals.				
3	0.2107	0.5013	0.1089	64.89	5.79
4	0.2161	0.5142	0.1072	64.89	5.55
5	0.2182	0.5205	0.1153	65.04	5.91
Calc. for $C_{30}H_{28}O_9.C_2H_4O_2$:				64.84	5.45
Calc. for $C_{30}H_{30}O_9.C_2H_4O_2$:				64.62	5.77

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[1] *Loc. cit.*

Analyses of other pure preparations of gossypol "acetate."

	Subst.	CO_2.	H_2O.	% C.	% H.
1.......................	0.1943	0.4628	0.0963	64.94	5.55
2.......................	0.2207	0.5257	0.1080	64.97	5.48
3.......................	0.2022	0.4819	0.0989	64.98	5.47

Pure "free" gossypol, prepared from the gossypol "acetate" (recrystallized 4 times) by removal of acetic acid and crystallization from diluted alcohol.

	Subst.	CO_2.	H_2O.	% C.	% H.
1.......................	0.2246	0.5573	0.1097	67.67	5.47
2.......................	0.2381	0.5929	0.1165	67.90	5.48
3.......................	0.2361	0.5861	0.1167	67.70	5.53

Free gossypol, earlier preparations, possibly less pure.

	Subst.	CO_2.	H_2O.	% C.	% H.
1.......................	0.1953	0.4878	0.0969	68.11	5.55
2.......................	0.2079	0.5183	0.1032	67.98	5.55
Calc. for $C_{30}H_{28}O_9$:				67.64	5.30
Calc. for $C_{30}H_{30}O_9$:				67.39	5.66

Compound of Gossypol with Acetone.

4.09 g. of pure gossypol was dissolved in ether and the ether removed by adding warm water. The crusts of gossypol left were redissolved in ether and the process repeated. The aqueous filtrate in each case was titrated separately with 0.1 *N* sodium hydroxide. The first filtrate required 68.8 cc. and the second 1.2 cc. of alkali to neutralize. The total—70.0 cc.—is equivalent to 10.27% acetic acid in the gossypol "acetate" used. The wet crusts were then dissolved in acetone and the solution concentrated. Beautiful glistening crystals separated, which were filtered out, washed with diluted acetone and dried in an exhausted desiccator. The analyses for carbon and hydrogen showed the substance was not identical with free gossypol. A weighed portion of the substance heated to 140–150° lost 1.0% in weight. Heated further to 200° it lost 16.24%. It thus differed from free gossypol, which loses about 7% in weight at 200°, and from gossypol "acetate" which loses 16.5–16.6% at this temperature. The vapors from heating the gossypol crystallized from acetone were passed through water which was found to give a positive result with Rothera's test for acetone. The substance is then a compound of gossypol with acetone.

Analysis of gossypol "acetonate."

Subst. 0.1532, 0.2240; CO_2, 0.3787, 0.5540; H_2O, 0.0808, 0.1181.

Calc. for $C_{30}H_{28}O_9.CH_3COCH_3$: C, 67.11; H, 5.81. Found: C, 67.41, 67.45; H, 5.77, 5.90.

Acetyl and Benzoyl Derivatives.

The acetyl and benzoyl derivatives of gossypol were made several times, but all attempts to obtain crystalline products failed. The acetylated products usually had a pale yellow tinge, although one product obtained in small amount from diluted alcohol was completely colorless. Gossypol is not quickly acetylated by acetic anhydride alone, but the addition of a drop of pyridine, or sulfuric acid or some fused sodium acetate causes the reaction to proceed very rapidly.

Benzoyl gossypol is readily made by the Schotten-Baumann reaction. The reaction product is rather difficultly purified. It was obtained as a whitish amorphous powder which was easily saponified, like the acetyl compound, with alkali or strong sulfuric acid.

The acetyl and benzoyl compounds were analyzed for carbon and hydrogen. The data for the particular acetyl derivative analyzed agree well for a tetra-acetyl gossypol. There is some evidence that more than 4 hydroxyls may be present for the results of the methods of determining the number of acetyl groups by direct weighing of the reaction product, and by hydrolysis of the acetyl derivative with strong sulfuric acid and subsequent weighing of the gossypol set free, indicate the probable presence of 5 hydroxyls.

The benzoyl compound gave results with the acid hydrolysis indicating 5 benzoyl groups, but the analyses for carbon and hydrogen agreed better for a lower benzoate. It is quite possible that some unexplained change may occur in the sensitive gossypol on acetylation or benzoylation.

Analyses of acetyl gossypol made, using pyridine as accelerator.

	Subst.	CO_2.	H_2O.	% C.	% H.
1	0.1648	0.3935	0.0806	65.13	5.48
2	0.1636	0.3912	0.0826	65.21	5.65
3	0.1655	0.3946	0.0822	65.03	5.56
Calc. for $C_{3},H_{24}O_5.(OCOCH_3)_4$:				65.11	5.18
Calc. for $C_{30}H_{26}O_5.(OCOCH_3)_4$:				64.93	5.45

Benzoyl gossypol made by Schotten-Baumann method.

1	0.1925	0.5126	0.0864	72.63	5.02
2	0.1320	0.3502	0.0593	72.35	5.03
Calc. for $C_{30}H_{24}O_5(OCOC_6H_5)_4$:				73.28	4.68

As the acetyl gossypol, like gossypol, is insoluble in water, it seemed possible to estimate the number of acetyl groups in the substance by simply weighing up the product precipitated in amorphous flocks from the acetylation mixture. In one case the pyridine method gave a product which weighed 41% more than the original gossypol after 8 hours' drying in the steam oven. A second experiment was conducted using 1.103 g. portions of gossypol "acetate" equal to exactly 1 g. of gossypol. One portion was dissolved in 10 cc. acetic anhydride and boiled for a few minutes after the addition of one drop of pyridine. The other portion was acetylated by boiling with 20 cc. of anhydride and 2 g. of fused sodium acetate. The excess anhydride was destroyed with alcohol and the acetylated products precipitated with water and collected, dried, and weighed from time to time. It was found impossible to bring the substances to constant weight for they continued to lose weight at the temperature of the water oven. The weights after the first and after the eighth drying are given below;

	(1) Pyridine—% increase.		(2) Sodium acetate—% increase.	
1st drying	1.3467	34.67	1.4288	42.88
8th drying	1.1965	19.65	1.2133	21.33

Calc. for $C_{30}H_{24}O_5(OCOCH_3)_4$: % increase in weight, 31.6%; calc. for $C_{30}H_{23}O_4(OCOCH_3)_5$: % increase in weight, 39.5%; calc. for $C_{30}H_{22}O_3(OCOCH_3)_6$: % increase in weight, 47.4%.

This loss of weight on prolonged drying at the temperature of the steam bath vitiates the accuracy of this method. The substance apparently is not stable at this temperature.

Analyses of the benzoyl and acetyl derivatives were made by dissolving them in concentrated sulfuric acid, warming slightly and allowing to stand overnight. The acid was then diluted with water to precipitate the gossypol. This was then collected in tared crucibles.

Percentage of gossypol found in acetyl gossypol.

1.... Subst., 0.2582 g. gave 0.1850 g. gossypol............ = 71.6%
2.... Subst., 0.1204 g. gave 0.0847 g. gossypol............ = 70.3%
Calc. for $C_{33}H_{24}O_5(OCOCH_3)_4$.......................... = 76.0%
Calc. for $C_{30}H_{23}O_4(OCOCH_3)_5$.......................... = 71.7%
Calc. for $C_{30}H_{22}O_3(OCOCH_3)_6$.......................... = 66.6%

Percentage of gossypol found in benzoyl gossypol.

1.... Subst., 0.0823 g. gave 0.0409 g. gossypol............ = 49.7%
2.... Subst., 0.0822 g. gave 0.0421 g. gossypol............ = 51.2%
Calc. for $C_{30}H_{24}O_5(OCOC_6H_5)_4$.......................... = 56.2%
Calc. for $C_{30}H_{23}O_4(OCOC_6H_5)_5$.......................... = 50.6%

Using a special modification of the method of Verley and Bolsing[1] for the determination of acetyl groups, it was found that, on *short* boiling in the presence of pyridine, gossypol apparently gives a tetra-acetyl derivative. The results corresponded to a 29.6% increase in weight due to addition of acetyl instead of 31.6% calculated for 4 acetyl, or 39.5% for 5 acetyl groups added. The reaction proceeds so smoothly that it was assumed, perhaps incorrectly that the gossypol is thus completely acetylated. The evidence is sufficient, however, to show there are 4 or 5 hydroxyl groups in gossypol.

Salts of Gossypol.

Gossypol readily forms salts with alkalies. It dissolves easily in aqueous sodium hydroxide, carbonate and very slowly in bicarbonate and disodium phosphate. Gossypol may be titrated as a dibasic acid with dilute alkali using phenolphthalein as an indicator. The alkali salts are very soluble in water and alcohol and are extremely sensitive to oxidizing agents. The yellow color of their solution soon turns greenish brown and then slowly to a beautiful blue which in turn gradually disappears. The alkali salts may be precipitated by saturated salt solution and a similar salting out action of the alkali is noted when attempts are made to break up gossypol with fused alkali.

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[1] *Ber.*, **34**, 3354, 3359 (1901).

Lime water and baryta water do not dissolve gossypol as readily as do alkali hydroxides, which seems to be due to the lesser solubility of these salts. Neutral salts of the alkaline earths do not precipitate gossypol from its alcoholic solutions, neither do silver mercury or copper salts. Iron sulfate and lead acetate give amorphous brownish and yellow lake-like precipitates, respectively, from alcoholic solutions of gossypol. Gossypol does not readily dissolve in strong or dilute ammonia water. The ammonium salt is probably formed but as an insoluble compound. After warming gossypol with dilute ammonia and cooling, the liquid formed a gelatinous mass, evidently due to the physical properties of the ammonium salt.

Compound of Gossypol with Aniline.

Gossypol dissolves readily in warm aniline with which it combines to form a bright orange-yellow substance,[1] which is possibly the dianiline salt of gossypol which separates out almost quantitatively on cooling the solvent. It is possible that instead of being a simple aniline salt of gossypol it may be a condensation product of carbonyl groups in gossypol with the amino group of aniline. The substance dissolves in alcoholic potash with a blood-red color which seems to persist for a period longer than one would expect if the alkali immediately replaced the aniline at the acid groups. Both "B" gossypol and "D" gossypol, described later, give similar orange-yellow precipitates.

The insolubility of the aniline compound of gossypol furnishes a method of determining the amount of gossypol in cottonseed products, and therefore a chemical method for determining the relative toxicity of a cottonseed product. These compounds do not dissolve readily in the usual organic solvents but are least soluble in petroleum solvents. Warm aniline dissolves them readily and they separate out in crystalline form on cooling. Alcoholic potash dissolves them with a beautiful red color which, however, disappears after the compound is decomposed by the reagent into gossypol and aniline.

The aniline compound of gossypol was first made by Dr. J. T. Dobbins

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[1] This substance heated to 100° was found to contain 3.97% nitrogen, which agreed sufficiently well with the 3.90% required for a substance of the formula $C_{30}H_{28}O_9.2C_6H_5NH_2$. This seemed at the time to be the most plausible explanation of the reaction since gossypol behaves as a fairly strong dibasic acid. Later this analysis was checked by another chemist, who reported 4.35 and 4.38% nitrogen on the unheated material. This appeared to indicate that aniline was disengaged on heating. I have more recently made analyses of this same preparation and have found 4.61, 4.64, 4.84, 4.70% nitrogen in the unheated substance. There is no perceptible odor of aniline to this substance. The average of 4.65% for the three most closely agreeing results agrees better for a compound containing 2 molecules of gossypol to 5 molecules of aniline, $2C_{30}H_{28}O_9.5C_6H_5NH_2$, which requires 4.58% nitrogen. My analyses of the aniline compounds "B" gossypol and "D" gossypol show, respectively, 4.84% and 4.15% nitrogen.

in this laboratory. It was desired to see if "B" gossypol, which at that time was thought to be the product in cottonseed meal formed from gossypol in the cooking process, could be made without the formation of the black material which accompanies it when gossypol is heated to 180–190°. It was thought by Dr. Dobbins that by heating the gossypol in aniline this could be avoided. It was found, however, that even in boiling aniline the gossypol was not decomposed as in dry heating. On cooling the solvent it was noticed that an orange-yellow crystalline substance was deposited. This interesting compound is not appreciably toxic, probably on account of its extreme insolubility. It passes through the digestive tract unchanged.

New Compounds of Unknown Formulas Derived from Gossypol.

Three substances which resemble gossypol more or less have been isolated. Two of these are yellow. The third was not isolated in a pure form but appeared to be without color. By heating of gossypol in air to its decomposition point a substance called for convenience "B" gossypol is formed, together with a black resinous substance. "C" gossypol is without color and is formed by fusing gossypol with alkalies to a fairly high temperature. "D" gossypol is the name given to a product isolated from cottonseed meal and which is thought to be the substance formed from gossypol in the cooking of the cottonseed. These three substances resemble gossypol in that they dissolve in strong sulfuric acid with the blood-red color characteristic of gossypol, and also oxidize in alkaline solution with the production of the beautiful blue color formed by gossypol.

Preparation of "B" Gossypol.—On heating gossypol or gossypol "acetate" to the decomposition point, 186–190°, these substances fuse with the evolution of vapor and then the melt solidifies to a black mass. This mass loses no more weight on heating to 220°. The loss in weight in case of dry free gossypol is about 6.5% and about 16.5% for the "acetate." This corresponds to a loss of two molecules of water from gossypol and two molecules of water and one molecule of acetic acid from the "acetate." If the melt is treated with limited amounts of ether, the black resinous matter passes into solution and a sparingly soluble yellow substance crystallizes out. This substance is more intensely yellow than gossypol and melts around 246–248°. It is much less toxic than gossypol. It is acidic like gossypol and has a neutralization value of about 300.

Preparation of "C" Gossypol.—In attempts to split gossypol into simpler known substances it was fused with alkali. In the concentrated alkali gossypol forms a yellow salt which is apparently salted out so that it does not mix readily with the melt. In one experiment the melt was heated more strongly than usual. It was dissolved in water, with the addition of a pinch of sodium hyposulfite to prevent oxidation. On acidifying a *white* flocculent precipitate formed instead of the usual yellow one.

The white precipitate was removed from the liquid with ether. The ether was evaporated to small volume and a little glacial acetic acid added. The new substance appeared to be very sensitive to oxidation for the solution rapidly became dark on standing. After some time a few crystals were formed. These appeared to be crystals of a colorless substance stained with brownish impurities. The substance dissolves in alkalies with an almost instantaneous formation of the blue color. On close examination, however, it was noted that this was preceded by a transient yellow-brown color. It is possible that the substance is closely related to the purple oxidation product of gossypol.

Preparation of "D" Gossypol.—The isolation of a gossypol-like substance from cottonseed *meal* furnishes an answer to the question of what becomes of gossypol in the cooking of cottonseed. Although gossypol may be easily removed from raw seed by ether, the same method applied to the meal failed to give an extract containing more than a trace of gossypol. The ether-extracted meal, however, gave a color reaction with alcoholic potash indicating the presence of much gossypol or a similar substance. It was earlier assumed that this was due to gossypol held perhaps dye-like by the seed tissue. The fact that gossypol combines with acetic acid and with aniline to form less soluble compounds suggested the idea that perhaps it was chemically combined with either carboxyl or amino groups of the protein. But the very slight toxicity for rats of cooked seed as compared with raw seed led to the conclusion that very little, if any, unchanged gossypol could exist in the cooked product. All attempts however to isolate this substance in the meal failed until it occurred to the writer to employ aniline as a solvent. It appears that under the influence of proper cooking conditions, conducted for a sufficient time all the toxic gossypol of the raw seed is changed to a substance which in the *meal* is so slightly toxic that on certain resistant species like the rat and fowl it has little or no effect if the diet is otherwise adequate.

The presence of this substance in considerable amounts in cottonseed *meal* accounts for the failure to find little or no gossypol-like substance in crude cottonseed oil (hot pressed) or in the ether extract of cottonseed meal.

To prepare "D" gossypol the cottonseed meal either before or after ether extraction is warmed to about 110° with enough aniline to make a thin paste. The meal turns to a deep brownish color. The aniline is sucked off on a Büchner funnel as much as possible, and the residue is washed with small amounts of ether until the filtrate is nearly colorless. The ether is driven off and the aniline solution allowed to stand for several days. A deposit of orange-yellow crystals appears after a day or so and gradually increases. The crystals obtained are often beautiful prisms over a millimeter long. This substance is the aniline compound of "D" gossypol. "D" gossypol was prepared from it by dissolving the substance in alcoholic potash and pouring into water. Oxidation was prevented by adding a pinch of sodium hyposulfite. The "D" gossypol was then precipitated in amorphous flocks by adding acid. The precipitate was taken up with ether. After evaporation of most of the ether, alcohol was added and the evaporation continued until a substance began to separate, after which a little more alcohol was added and the warm mixture allowed to cool. A crop of yellow crystals was secured. The substance showed no well-defined melting point but darkened and softened around 256°.

Unfortunately the author was not able to do more work on this substance. The method for obtaining it was worked out just before he left the experiment station. Certainly more work should be done on this substance, which occurs to such a considerable extent in cottonseed meal.

From a 100 g. sample of a meal cooked 28 minutes the aniline extraction, after previous ether extraction, gave 1.2 g. of the aniline salt of "D" gossypol. About one g. of the aniline compound was obtained from 100 g. of a meal which had been cooked 2 hours. Qualitative tests on cottonseed flour also showed a considerable amount present.

Since the food-products examined were definitely injurious to rabbits and pigs and since no gossypol *as such* could be found in these products, these facts indicate that this "D" gossypol gives rise to cottonseed-meal poisoning of rabbits and swine. The substance has so little action on rats that it is not possible to state whether the animals are affected by an active substance or are suffering from nutritive deficiencies. There are then chemical reasons for believing that cottonseed meal poisoning of swine may be due to the presence of unchanged gossypol, or to "D" gossypol, or to the presence of both.

Action of Nitric Acid on Gossypol.

Strong nitric acid dissolves gossypol with evolution of nitric peroxide fumes and with formation of a substance no longer giving the color reactions of gossypol. The product is soluble in alkalies with a reddish yellow color and is precipitated by acids. It could not be obtained in crystalline form.

Oxidation of Gossypol in Alkaline Solution.

Alkaline solutions of gossypol oxidize readily on exposure to air or more readily by adding hydrogen peroxide. This oxidation proceeds in several stages. A blue substance is formed first which may be reduced by alkaline hyposulfite to the yellow gossypol. The blue stage subsequently disappears and a more stable oxidation product is obtained which has little coloring power. This substance can not again be reduced to gossypol. It is precipitated by acids and dissolves in alkalies. Many attempts to obtain it crystalline were without success. The substance seemed to be rather complex. It resembled gossypol in its solubility in the solvents.

Chemical Constitution.

Attempts to get a clue to the constitution of gossypol have failed through inability to split the substance into simpler known substances.

The fact that several flavone pigments occur in the cotton plant and the fact that gossypol has 30 carbon atoms suggests that it may be derived by condensation and subsequent reduction of two molecules of a flavone. The acidic properties are thought to be due to carbonyl and hydroxyl group arranged as in flavonols rather than to carboxyl groups. These substances are sufficiently acid to form salts from an alkali acetate. The presence of *o*-hydroxyl is indicated by the green ferric chloride reaction and by the formation of lake-like compounds with lead and ferrous salts. The presence of 9 oxygen atoms may be readily accounted for by the presence of 5 hydroxyls, 2 carbonyl groups and 2 bridge oxygen atoms, all of which types occur in the flavonols.

Presence of Gossypol as such in Commercial Cottonseed Products.

Examination of a number of cottonseed products with a view to determining the fate of gossypol has given results which are of considerable interest. Very little, if any, gossypol was found in samples of hot pressed meal. In one case a small amount was found where the seed used was very dry. On the other hand in the so-called "cold pressing" process at least three-fourths of the gossypol of the seed passed into the oil. Such an oil was found to contain 1.5% gossypol. On treatment with alkali any gossypol in the crude oil is quantitatively removed and passes into the foots. Since crude oil is always treated with alkali no gossypol or related pigment is ever found in the refined edible oil. Since the non-occurrence of gossypol in hot pressed oil is contrary to what might be expected and since it does not occur in *actually cold pressed* oil it may be well to explain the difference. Actual cold pressure squeezes out the oil from the oil cells without allowing it to dissolve gossypol from the "resin glands." Hence the gossypol remains in the press cake. In the commercial "cold pressing" process the seed is heated to a considerable extent and the material is subjected to a grinding, pulverizing action under great pressure so that the glands are broken up and the contents taken up by the hot oil and removed mostly from the cake.

In the hot pressing process as ordinarily conducted under the moist cooking conditions the gossypol glands are disintegrated by the moisture and stirring and the contents stream out and are spread over the seed tissue, where the gossypol is subjected to oxidizing influences. It is not clear why so little passes into the oil, but perhaps the seed tissue holds the gossypol and its oxidation product—"D" gossypol— much as cloth holds a dye, or possibly some chemical combination may take place.

If rather dry seed is used the gossypol is apparently not so readily converted to this less soluble, less toxic oxidation product but remains in part as such in the meal. Such a meal is more toxic than a properly cooked meal. In these cases gossypol may be extracted with ether and the amount estimated by the aniline method. It is interesting to note that gossypol in crude oil behaves much the same as free fatty acid. This was shown by dissolving some gossypol in neutral cotton oil, after which alkali was required to render the oil again neutral to phenolphthalein.

Thus the presence of a considerable amount of gossypol in crude oil would increase the refining loss. The writer has been informed that commercial "cold pressed" oil tends to show a smaller refining loss than hot pressed oil. Provided the same seed were used in each process the author believes that the reverse would be true owing to the presence of considerable amounts of gossypol in the crude cold-pressed oil.

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STUDIES ON THE BACTERIAL METABOLISM OF SULFUR.

II. FORMATION OF HYDROGEN SULFIDE FROM CERTAIN SULFUR COMPOUNDS BY YEAST-LIKE FUNGI.

BY FRED W. TANNER.

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In a series of studies which are being made on the relation of sulfur to bacterial metabolism, it seemed important to investigate this question with regard to the yeasts. Even though these microörganisms are not bacteria, the report of this investigation is included in the series. In a former paper, the author[1] has shown that bacteria are important in forcing sulfur through its cycle. Little data are available in the literature with regard to the relation of sulfur compounds to the metabolism of yeasts. A great part of that which does exist must be taken from reports of investigations which are directly concerned with other things. Lafar[2] who has given a fairly complete résumé of the work on sulfur has stated that the sulfur metabolism of yeasts is very much in the dark.

Small amounts of sulfur are probably necessary in the nutrition of the budding fungi. Ash analyses of yeasts made by different investigators are reported by Lafar[2] which show a variation in sulfur trioxide content of from 0 to 6.38 per cent. Since sulfur has been found in most yeasts, it seems reasonable to assume that it may be closely connected with their metabolism.

De Rey-Pailhade[3] reported the formation of hydrogen sulfide by yeasts and believed that its formation was due to the reducing enzyme which he called *philothion*. These data were later verified by Sostegni and San-

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[1] Tanner, F. W., *Jour. Bact.*, **2**, 585–593 (1917).

[2] Lafar, F., *Handbuch der Technischen Mykologie*, **4**, 83–84 (1904).

[3] De Rey-Pailhade, M. J., *Bull. soc. chim.*, [3] **23**, 666–668 (1900).