The Determination of Gossypol Structure

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THE next phase of the problem undertaken was that of obtaining information which might lead to the determination of the structure of gossypol. Work of this nature has previously been limited to the experiments of Carruth (3) who studied the effects of alkali fusion and several oxidizing agents upon gossypol but did not succeed in obtaining any pure crystalline derivatives or other evidence of a clarifying character.

Among many possibilities which presented themselves was the action of hot concentrated alkali upon gossypol (13). This reagent, forty per cent sodium hydroxide, at the temperature of the steam bath, brought about a well defined transformation. When the two components are first brought together, the gossypol does not immediately dissolve as it does in less concentrated alkali, but forms a dark orange paste. If this is continuously heated at the temperature of the steam bath and frequently stirred, a reaction slowly takes place in which the color of the mixture becomes gray.

The products of the reaction are formic acid and a new phenolic substance which has been designated as apogossypol. Apogossypol is a colorless crystalline material having no definite melting point. It is soluble in ordinary organic solvents, and the solutions thus formed darken more or less quickly depending upon the nature of the solvent employed. It dissolves freely in dilulte alkali, from which it is precipitated by carbon dioxide. Its alkaline solutions darken at once and rapidly acquire a purple color. Even crystalline apogossypol is so unstable that a few hours' exposure of the dry crystals to air and light causes them to change to a jet black powder. Another characteristic of its phenolic nature is the bluish green color reaction produced when a dilute alcoholic solution of the substance is treated with ferric chloride.

Apogossypol Derivatives

Apogossypol readily forms colorless crystalline acetyl and methoxy derivatives, which are, in contrast with the free substance, quite stable. A study of these substitution products, as well as of the free phenol, has shown that apogossypol has the molecular formula $C_{28}H_{30}O_6$. Furthermore, it has been shown that there are six acetyl groups in the acyl derivative. No direct determination of the methoxy groups in the methyl ether could be made since the substance was completely inert in boiling hydriodic acid under the conditions used in the Zeisel method. Carbon and hydrogen figures, however, checked well for a hexamethyl ether. It therefore follows that all the oxygen atoms in apogossypol are present as hydroxyl groups. From a consideration of the molecular formula for gossypol, $C_{30}H_{30}O_8$, it will be seen that strong hot alkali, under the conditions of the

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*Continued from the August issue of OIL AND FAT INDUSTRIES.

experiment, functions only in removing the two carbonyl groups from the gossypol molecule. Since formic acid is the only other substance produced by the reaction, the carbonyl groups must have been eliminated as formic acid. Such a process requires that each mol of gossypol should produce one mol of apogossypol and two mols of formic acid. The yield of each of these substances actually obtained corresponded to 97.5 per cent and 95.3 per cent respectively of that demanded by the theory.

Nature of Chromophores

It is noteworthy that in the elimination of the two carbonyl groups from gossypol the characteristic yellow color of the substance disappears. This strengthens the hypothesis previously advanced that the chromophores of gossypol are carbonyl groups.

The toxicity of apogossypol was found to be approximately one third of that of gossypol. The lethal dose, when given intraperitoneally in oil solution to white rats, is from sixty to seventy-five mg. per kilogram of body weight. Its physiological action, however, is in sharp contrast with that of gossypol as no chronic effects follow the administration of small doses. Its action seems to be restricted to the development of acute toxic symptoms only.

Experiments upon the oxidation of gossypol (14) as a whole have been more or less discouraging, for with most reagents and methods tried either nothing or only amorphous or tarry products, having no tendency to crystallize, were obtained. With potassium permanganate, however, certain products were obtained that have a significant bearing upon at least one phase of the problem. In these experiments gossypol dissolved in slightly more than 2 mols of sodium hydroxide was treated at 0° with potassium permanganate in the proportions of one part of gossypol to five parts of permanganate. The reaction was decidedly energetic and terminated in a short time. The manganese dioxide formed was removed by filtration, and the filtrate was made acid with sulfuric acid. In adition to the evolution of carbon dioxide there was evidence of the presence of volatile fatty acids in the reaction mixture. The solution was therefore steam-distilled, and the distillate was examined by the method of Dyer (15) for fatty acids. From an examination of the distillation curve obtained it was possible to show that formic, acetic and isobutyric were the acids present in the mixture. These conclusions were verified by applying specific confirmatory tests and by preparing certain characteristic derivatives of the respective acids. It was further demonstrated that the acids mentioned were the only ones present. A quantitative estimation of the different acids was made, and the yield obtained in the case of isobutyric acid was 92.5 per cent of that required by the theory if it is assumed that each mol of gossypol yields one mol of isobutyric acid.

Evidence Supporting Side-Chain Theory

The existence of formic acid under the conditions of the experiment may appear at first to be paradoxical; but when it is considered that the potassium permanganate was not used in excess, it is evident that some of this acid could easily escape oxidation. It would be unwise at this time to attribute the formation of formic and acetic acids to any definite structure in the original material, but the identification of isobutyric acid as a decomposition product seems to indicate that there is a side chain in the gossypol molecule involving at least the group $(CH_3)_2{=}CH{-}\overset{1}{\underset{1}{C}}{-}$.

Oxidation Products

From the results obtained in attempting to oxidize gossypol it was soon realized that in order to progress in the degradation of the molecule, the hydroxyl groups would have to be protected. There are available at the present time three derivatives fulfilling this condition, namely, hexaacetyl gossypol, hexaacetyl apogossypol and apogossypol hexamethyl ether. These materials were subjected to oxidizing agents under various conditions and to date three different products have been obtained in analytically pure crystalline form. The oxidizing agent used in the preparation of each was chromic acid, but the conditions imposed in the various cases were different.

The product obtained from hexaacetyl gossypol is a bright yellow neutral compound which possesses no carboxyl or free hydroxyl groups and which has the molecular formula $C_{33}H_{30}O_{12}$. It is a tetraacetyl derivative of a substance $C_{25}H_{22}O_8$. It follows that in its formation two acetyl groups have been replaced by two quinone groups and that the substance has a smaller molecular weight by C_5H_8 than gossypol. The carbonyl groups originally in gossypol apparently have been retained in the new substance, as aniline condenses with it in much the same manner as with gossypol. This is not the case with the quinones to be immediately described which are obtained from apogossypol.

A product obtained by the chromic acid oxidation of hexaacetyl apogossypol is a bright yellow neutral substance having a melting point of 230° and the molecular formula $C_{30}H_{28}O_{10}$. It is a tetraacetyl derivative of a compound $C_{22}H_{20}O_6$. In this case two acetyl groups have also been replaced by two quinone groups, and the original apogossypol has lost C_6H_{10}.

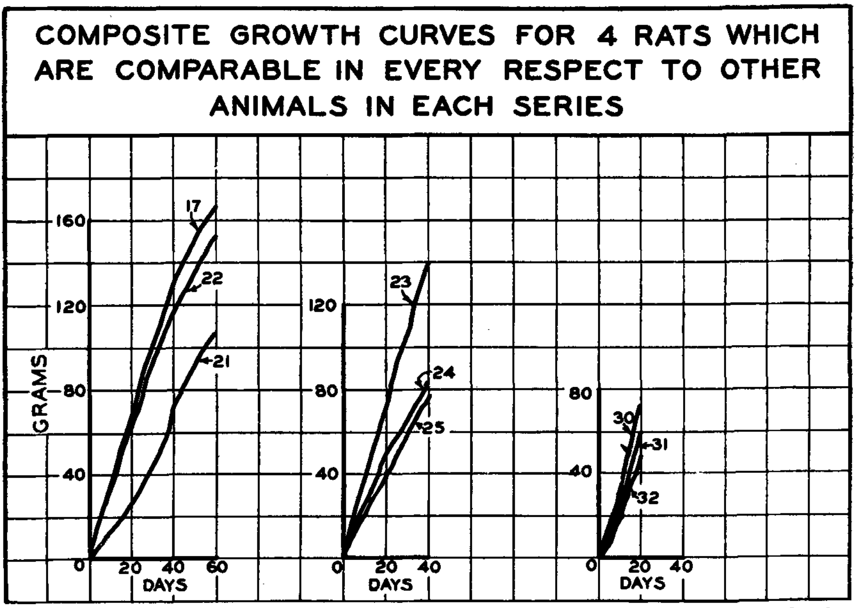
The oxidation of apogossypol hexamethyl ether gave a bright yellow neutral crystalline compound $C_{32}H_{34}O_8$. It was impossible to determine the methoxy groups in this preparation, as boiling hydriodic acid under the conditions used in the Zeisel method had no appreciable action upon the substance; but from analogy it appears that two methoxy groups have been replaced by quinone groups and at the same time two hydrogen atoms have been oxidized to quinone groups. If this is the correct explanation of the mechanism of the reaction, the new substance would be a tetramethyl ether of a tetra quinone $C_{28}H_{26}O_8$.

Biological Experiments

The foregoing information summarizes the definite chemical facts thus far obtained. The nature of the results precludes the possibility of making a statement regarding the nature of the gossypol molecule; but the experience gained by working with the material has indicated a method by which the problem of cottonseed injury may be attacked. Work in this direction is now in progress, but as it is necessary in such investigations to resort to animal experimentation, attention was directed to the choice of suitable animals for the purpose. Since the Protein and Nutrition Division of the Bureau of Chemistry and Soils, United States Department of Agriculture, has been engaged in and is equipped to carry on such studies, their cooperation, in the personnel of Dr. E. M. Nelson and Dr. D. B. Jones, was secured.

In this work, as in the study of nutritional problems in general, it was desirable to employ small laboratory animals for the necessary tests. The use of such animals would offer the advantage of economy in labor and expense; and also because of the character of the material to be investigated, their use would insure more dependable results. The albino rat is the animal best suited for this purpose, but certain workers have conveyed contradictory impressions regarding its susceptibility toward orally administered gossypol.

Our first problem was therefore to establish to what extent results obtained on rats could be applied to gossypol poisoning studies. Some experiments along this line will be briefly described. Five groups of rats received a basal diet containing graduated quantities of pure gossypol; a sixth group, used



COMPOSITE GROWTH CURVES FOR 4 RATS WHICH ARE COMPARABLE IN EVERY RESPECT TO OTHER ANIMALS IN EACH SERIES

Each curve represents the composite growth rates of 4 rats. At the beginning of the experiment all the animals weighed approximately 40 gm.

17—Controls on basic ration.

21—Basic ration plus 0.1 per cent gossypol.

22—Basic ration plus 0.05 per cent gossypol.

23—Controls.

24—40 per cent raw cottonseed flour, gossypol free.

25—40 per cent cottonseed meal flour, 1.6 per cent bound gossypol.

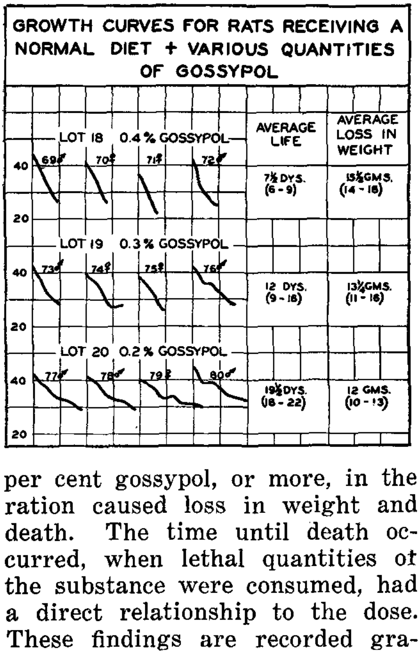
30—Controls.

31—40 per cent raw cottonseed flour, gossypol free, with modified salt mixture.

32—40 per cent cottonseed meal flour

as controls, received the same diet without gossypol. The animals in all the groups were comparable as to weight, sex, and parentage, and the basic diet was adequate in every known factor necessary for an optimum rate of growth.

The growth curves were surprisingly uniform and showed that even 0.05 per cent of pure gossypol produced a noticeable retardation of the growth rate. One tenth of one per cent gossypol produced much greater depressions, and 0.2 per cent gossypol, or more, in the ration caused loss in weight and death. The time until death occurred, when lethal quantities of the substance were consumed, had a direct relationship to the dose. These findings are recorded graphically in the accompanying charts.



Animals receiving a ration in which forty per cent of the normal diet was replaced by cottonseed flour to serve as the sole source of protein grew more slowly than the controls. In one experiment raw cottonseed flour freed from gossypol was used, and in another experiment cottonseed meal flour containing no free gossypol, but having 1.6 per cent bound gossypol was fed. The animals on the raw cottonseed diet grew a little more rapidly than those on the cottonseed meal preparation. The difference between the growth rates of animals receiving cottonseed flour and the controls was in part due to an improper mineral balance. When this factor was corrected in another set of experiments the growth rates of the animals on the modified diets were better than those of the previous set but they were still below those of the controls. Here again the animals receiving cottonseed meal made the least progress. These results are shown by the accompanying curves.

Although the technique of testing the toxicity of cottonseed meal products has not yet reached the desired degree of perfection, it is hoped that practical information resulting from the chemical data now available upon gossypol will soon be forthcoming.

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