Studies on Gossypol: A Progress Report

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DURING the past year, work upon the study of cottonseed meal and gossypol has continued along the lines indicated in the report presented to this Society at the annual meeting here last May[1]. Some of the results obtained in this investigation are summarized in the following outline.

In the course of the year's work a new supply of gossypol had to be prepared, and because of greater familiarity with the properties of the phenol, it was possible to devise a more economical and convenient method for its preparation. Since the availability of gossypol for future work is important the improved procedure for its preparation is given. Ten kg. ground cottonseed are percolated during 3 hours with sufficient ether to yield approximately 30 liters of extract. The ether is recovered by distillation, and the residue is filtered through activated carbon. The filtrate is dissolved in an equal volume of petroleum ether and then treated with the same volume of acetic acid. Crystallization of the so-called acetate begins at once, and the process is complete within a few hours. The yield corresponds to approximately 90 per cent of that in the seed, and the product is much purer than the preparation made by the method previously reported[2].

In continuing the oxidative degradation studies of gossypol, the products obtained by chromic acid oxidation of certain gossypol derivatives referred to in the report mentioned before have been further investigated[3]. These derivatives are hexaacetyl gossypol, hexaacetyl apogossypol and apogossypol hexamethyl ether.

When dissolved in acetic acid and oxidized with an aqueous solution of chromic acid, hexaacetyl gossypol yields a bright yellow neutral compound which begins to darken at 210° and becomes a black mass at 230° without definitely melting. This substance has the molecular formula $C_{33}H_{30}O_{12}$. It possesses no carboxyl or free hydroxly groups but has four acetyl groups and is therefore a tetraacetyl derivative of a substance, $C_{25}H_{22}O_8$, to which the name *gossypolone* has been given. The derivative obtained is therefore tetraacetyl gossypolone. From the foregoing facts it follows that, in the formation of tetraacetyl gossypolone from hexaacetyl gossypol, two acetyl groups are replaced by two quinone groups, and carbon and hydrogen are lost in such proportions that gossypolone has a smaller molecular weight than gossypol by C_5H_8. Tetraacetyl gossypolone readily condenses with aniline in much the same manner as does gossypol. The condensation product is a chocolate colored micro-crystalline substance which imparts a deep wine red color to solvents capable of dissolving it. It would appear from this that the two carbonyl groups originally in gossypol have not been materially affected in the new substance.

When hexaacetyl apogossypol is treated in the same manner as hexaacetyl gossypol, except that Kiliani's chromic acid mixture[4] is used, a substance similar in appearance to tetraacetyl gossypolone is obtained. This is a neutral material having a melting point of 230° and a molecular formula of $C_{30}H_{28}O_{10}$. It has been shown to be a tetraacetyl derivative of a compound $C_{22}H_{20}O_6$. The name assigned to this substance is *apogossypolone*. In the reaction by which this material is formed, as in the previous one, two quinone groups have been substituted for two acetyl groups, and the new nonacetylated derivative has a smaller molecular weight than apogossypol by C_6H_{10}. One property of this new acetyl derivative which distinguishes it from tetraacetyl gossypolone is its inability to condense with aniline.

The oxidation of apogossypol hexamethyl ether with chromic and sulfuric acids (Kiliani's mixture) gives a bright yellow neutral crystalline compound, $C_{32}H_{34}O_8$, having a melting point of 210°. It has been established that this material is a tetramethyl ether of a tetraquinone, $C_{28}H_{26}O_8$. The name *pseudogossypolone* has been assigned this substance, and hence the C_{32} compound isolated is *tetramethoxy pseudogossypolone*. The process by which it is formed is analogous to the reactions involving the acetyl derivatives discussed before, in that two quinone groups have been substi-

[1] Presented before the American Oil Chemists' Society at New Orleans, La.

tuted for two methoxyl groups. On the other hand, methoxy derivatives of gossypol in general are more resistant to reagents than the corresponding acetyl derivatives. In this experiment chromic acid did not attack the apogossypol molecule in such a manner as to cause a loss of carbon. Two quinone groups, however, were substituted for two hydrogen atoms.

Further oxidation of the acetyl derivatives of gossypolone and apogossypolone with potassium permanganate were successful only in that they showed that no isobutyric acid was formed as is the case with gossypol. No crystalline derivatives were obtained. However, the oxidation of pseudogossypolone tetramethyl ether with potassium permanganate gave two crystalline acids and evidence of isobutyric acid. The first acid obtained, which has been designated as apogossypolic acid, is a white crystalline substance that begins to sinter at 140° and melts with the evolution of gas at 164°. It is a trimethyl ether of a tricarboxylic acid, $C_{20}H_{24}O_9$. When heated to the temperature of its melting point it loses one molecule of water, giving a new acid, $C_{20}H_{22}O_8$, which melts at 95°. This acid appears to be a trimethyl ether of a ketonic dicarboxylic acid; but further work is necessary to verify this point.

The second acid referred to above is obtained from the mother liquors from the first acid. It is an orange crystalline product which melts at 116°. It appears to be a dicarboxylic acid produced as an intermediate stage in the formation of apogossypolic acid; but since the work upon this substance has not been completed, a definite statement concerning it will have to be withheld.

The fact that isobutyric acid is not found as a permanganate oxidation product of the acetyl derivatives of gossypolone and apogossypolone indicates that the loss of the elements C_5H_8 and C_6H_{10} in these reactions is due in part to the breaking of a ring to which is attached an isopropyl side chain. It has previously been pointed out[5] that one of the products of the permanganate oxidation of gossypol is isobutyric acid and further, that this indicates the presence of a group $-\overset{|}{\underset{|}{C}}-CH<^{CH_3}_{CH_3}$ in the gossypol molecule. Since no definite evidence of an ethylene linkage could be obtained, the isobutyl group as indicated above is very possibly an isopropyl side chain attached to a ring thus: $\overset{\wedge}{\underset{\vee}{C}}(=C)-CH<^{CH_3}_{CH_3}$ The rupture of the ring could reasonably be expected to yield isobutyric acid.

Another approach to the structural relationships existing in the gossypol molecule has been attempted through a Beckmann rearrangement of the dioxime. The reaction, however, is not simple, and, although a number of promising derivatives have been obtained, no definite results can be reported at this time, since the study of these compounds has not yet progressed to a point where the data available can be correlated. It is hoped that at a future time it will be possible to report upon this work.

Biological Experiments With

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IN the report referred to before some biological experiments were presented showing that white rats respond readily to the toxic action of gossypol and hence may be employed for studying the physiological effect of this important phenol. Curves were also shown, representing the influence on the growth of rats fed upon a normal diet to which gossypol had been added in quantities sufficient to cause death in a relatively short time.

In continuing this work the effect of feeding lower levels of gossypol upon the growth rates of rats has been studied, and data are now available showing the effect upon rats of diets containing proportions of gossypol ranging from levels which only slightly influence the growth rate to quantities capable of producing death in a short time. The results of these studies are given in the form of curves on Charts 1, 2, and 3.

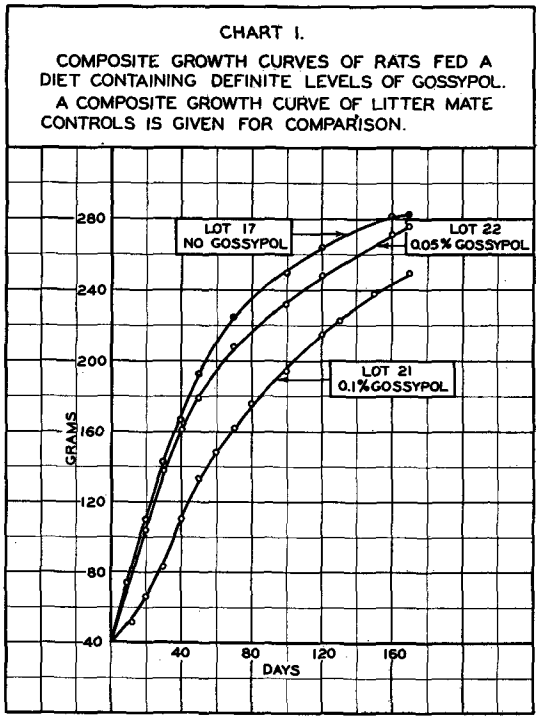

Fig. 1

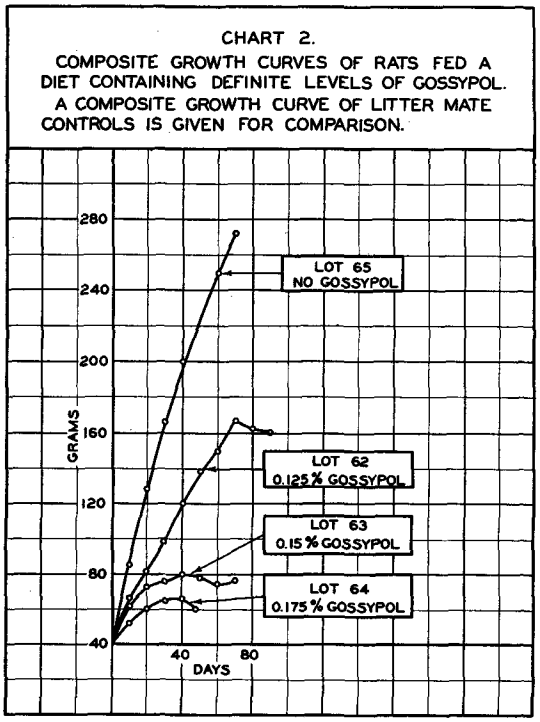

Fig. 2

Each graph except on Chart 3 represents the composite growth curve of 4 animals comparable as to age, sex and parentage. The curves

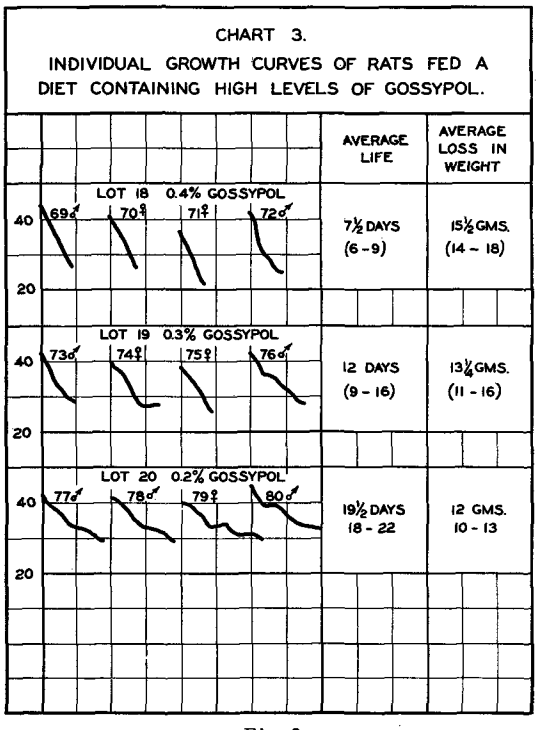

Fig. 3

on Chart 1 show that the growth rate of rats is retarded when the animals receive low levels of gossypol and that the retardation is proportional to the quantity of gossypol in the diet. The maximum difference in average weights between the control lot and the lots fed low levels of gossypol occurred at 80 to 90 days, or at about the end of the rapid growing period of the controls.

Chart 2 shows the sensitivity of rats to small differences in the quantity of gossypol in their diet. Lot 65 was discontinued at the end of 70 days; the animals in Lot 62 were alive, but losing weight after 105 days. The termination of the curves for Lots 63 and 64 indicates the point at which one animal in the

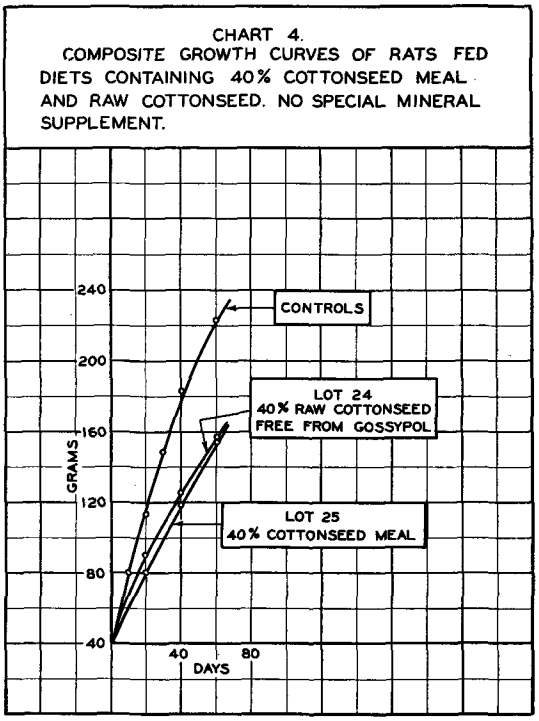

Fig. 4

respective group died. Three animals in Lot 63 died at 70, 76, and 99 days respectively, and at the end of 105 days the remaining animal was emaciated and losing weight rapidly. In Lot 64 the life of the rats ranged from 46 to 99 days, the average being 67 days.

The results represented by Chart 3 show remarkable uniformity in the response of the animals to the effect of pure gossypol when it is fed at decidedly toxic levels. As the quantities of gossypol in the diet were decreased, the period of survival increased and the daily loss in weight lessened. On the 0.4 per cent level the average daily loss of weight was 2 grams; on the 0.3 per cent level it was 1.1 gram

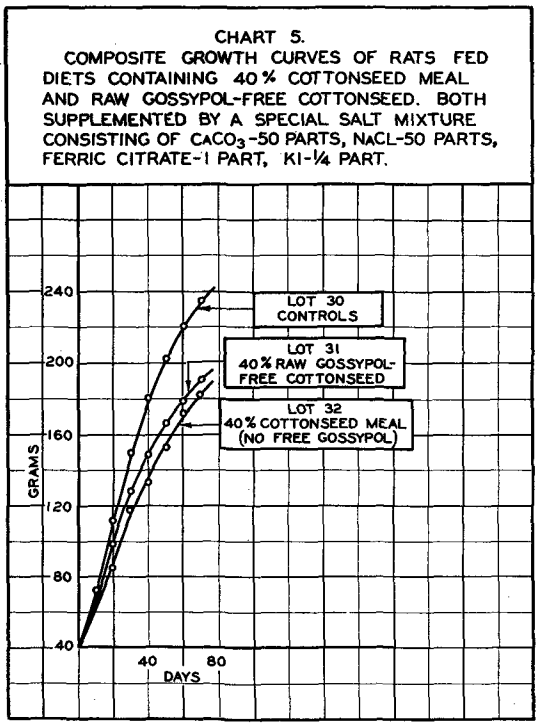

Fig. 5

and on the 0.2 per cent level it was 0.6 gram. Lot 17 in Chart 1 shows the performance of litter mate controls on a gossypol-free diet.

In the previous report it was indicated that when the protein of a diet is supplied entirely by cottonseed meal flour, or raw cottonseed free from gossypol, the ash content of the cottonseed products influences the growth rate of the animals. Experiments bearing upon this point have been continued, and the results are presented graphically on Charts 4 and 5. From an analysis of the curves it will be seen that a 1 per cent mineral supplement consisting of calcium carbonate—50 parts; sodium chloride—50 parts; ferric citrate—1 part, and potassium iodide—¼ part, added to the cottonseed ration, increases the growth rate of the animals approximately 15 per cent.

It is to be noted, however, that the growth rate of the animals fed upon the cottonseed products, even when supplemented by the salt mixture, is decidedly inferior to that of the controls. Attention is also directed to the slightly superior growth rates of the animals fed upon a ration containing raw, gossypol-free cottonseed as compared with the growth rates of the animals fed upon a cottonseed meal ration.

In looking for an explanation for the inferior growth rate of the animals fed upon a salt supplemented cottonseed ration, as compared with the growth rates of controls on a normal diet, attention was directed to the influence of a protein supplement. In the experiments represented by Chart 6 the same diets were fed as in the experiments recorded by Chart 5, except that 6 per cent casein was added. The curves of the two charts show that the growth rate of the animals fed the casein supplemented diet was superior to that of the rats on a diet that contained no casein. The same influence is shown by the experiments recorded on Charts 7 and 8. In addition the results recorded by Chart 7 show the physiological effects of cottonseed meals prepared by different commercial processes. Cottonseed meal No. 1 (an Expeller meal) contained no free gossypol, and, when supplemented by 6 per cent casein, gave a growth rate almost the same as that of the control animals; the addition of 12 per cent casein gave identical growth rates. The difference in average weight between Lots 45 and 49 was never more than 7 grams and during 80 per cent of the experimental period it was 4 grams or less. Cottonseed meal No. 2, which was a special process meal, showed a demonstrable toxicity. The retardation of growth corresponded to that which would be produced by approximately 0.05 per cent free gossypol. A

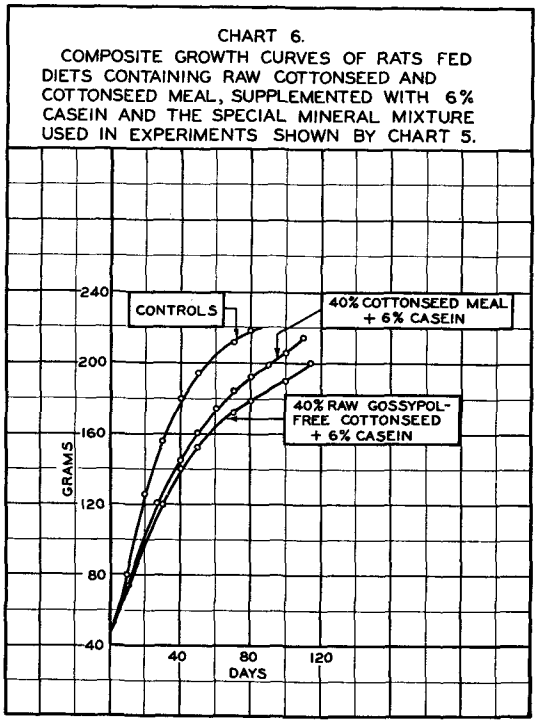

Fig. 6

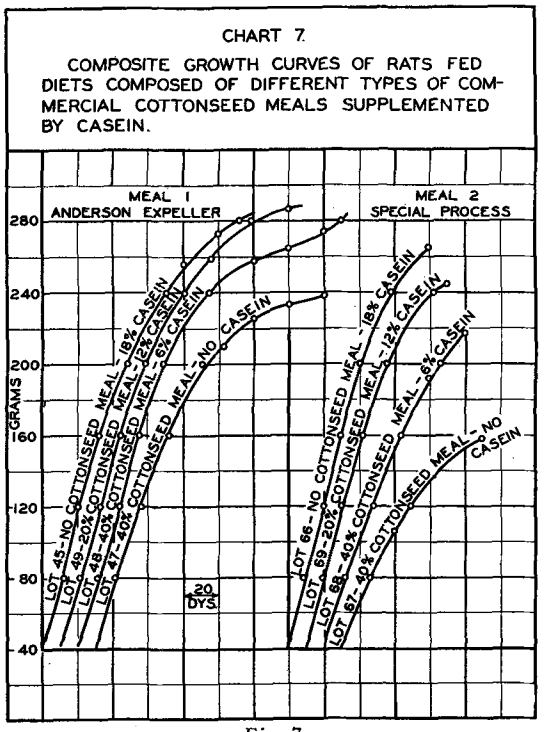

Fig. 7

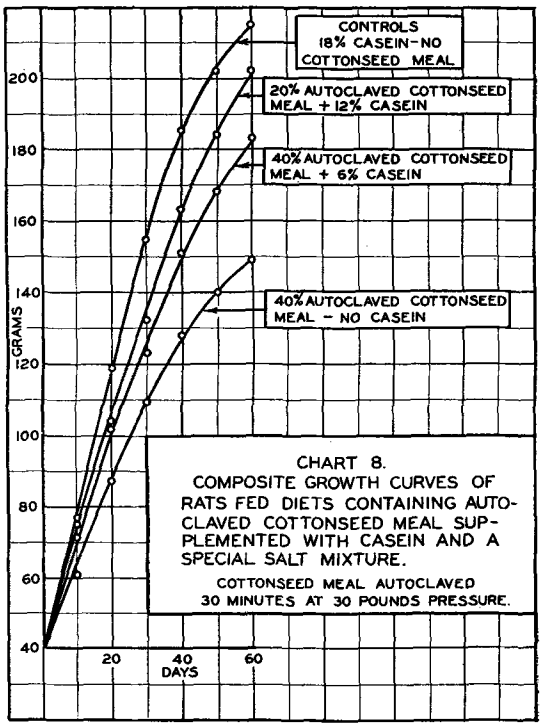

Fig. 8

chemical analysis showed the presence of .06 per cent.

Chart 8 is presented not only to show the influence of casein, but also to verify the claims made concerning the beneficial effects of autoclaving cottonseed meal according to the Menaul process.[6] The meal used in these experiments was the same as used in the experiments recorded by Chart 5. Comparison of the two charts will show that, in spite of the claims made,[7] the growth rate of rats fed on the autoclaved cottonseed meal is inferior to that attained by the rats fed upon the unautoclaved meal.

Further work along the plan just outlined, as well as other phases of the problem, is necessary, but the results thus far obtained warrant the following conclusions:

1. White rats respond readily to the toxic action of gossypol, and they may be used as an accurate means of determining physiologically the free gossypol content of cottonseed meal.

2. Growth rates of rats fed upon diets containing high levels of cottonseed meal are accelerated by a protein supplement. The growth rates are also influenced by the composition of an added mineral supplement.

3. The results shown by chart 7 indicate that *bound gossypol per se* has no influence upon the growth rate of rats. This conclusion follows from the fact that rats grew normally on diets containing *bound gossypol* equivalent to 0.4 to 0.6 per cent of free gossypol.

4. Growth curves of rats fed upon a diet containing autoclaved cottonseed meal prepared according to the Menaul process show that feeding the autoclaved meal resulted in growth rates inferior to those obtained with untreated meal.

In applying these findings to practical live stock feeding, it would appear that the proper method of using cottonseed meal would be as a protein supplement to other feedstuffs. Such a mixed feed should be balanced so that a proper ratio exists between the protein, carbohydrate, fat and mineral constituents. Since the proteins of the components of the mixed feed, other than cottonseed meal, would undoubtedly function in the same manner as does casein in the experiments just recorded, there is little doubt that, by an intelligent use of cottonseed meal as a protein supplement to a mixed feed, an excellent stock ration should result.

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