is probable that formaldehyde is closer to the immediate precursor of the β-carbon of serine than either formate or methanol.

*Summary.* $C^{14}$ from labeled formaldehyde was incorporated into the β-carbon of serine greater than that from labeled formate or methanol. The indications are that formaldehyde is the more direct precursor of the β-carbon of serine.

*Addendum.* As an extension of the work by Welch and Sakami(18) in which they found that in the intact rat and in liver slices there was incorporation of $C^{14}$ from formate into the methyl groups of methionine and choline we have made some preliminary studies with $C^{14}$-formaldehyde. Employing the same rat liver homogenate used in these serine studies we have found evidence that formaldehyde is converted into the methyl group of methionine much more extensively than is formate.

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## Preparation of Water-Soluble Combination Products of Gossypol and their Toxicity to Aquarium Fish.* (17996)

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The purpose of the investigations here described has been to make combination products of gossypol(1-3) with naturally occurring materials which would have physical properties different from those of the original material and to determine the effect of such changes on some physiological action of gossypol. Gossypol was tested on aquarium goldfish and sharp differences in physiological activity between the original gossypol and the combination products were obtained.

*Materials and methods.* Pigment glands were prepared from cottonseed by the gland fractionation process(4,5). Gossypol was prepared by acetone extraction of pigment glands and crystallization from a mixture of diethyl ether and light petroleum naphtha (6). Peanut protein was prepared by alkaline extraction of oil-free peanut meal followed by acid precipitation of the protein (7). The casein, amino acids, dextrose, and starch used in the experiments were commercial products.

The general procedure for preparation of the combination products involved two steps, namely: (1) Gossypol and the material to which it was to be combined were solubilized in aqueous solution of pH of approximately 11 in the shortest possible time. (2) The solubilized material was neutralized with hydrochloric acid and dried in the frozen state (lyophilized).

Ten grams of purified peanut protein was added to one liter of distilled water and the

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† One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

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7. Arthur, J. C., Crovetto, A. J., Moliason, L. J., Guilbeau, W. F., and Altschul, A. M., *J. Am. Oil Chem. Soc.*, 1948, v25, 398.

pH was adjusted to 8.5 by use of dilute aqueous sodium hydroxide. While the solution was being stirred 10 g of gossypol and more sodium hydroxide were added until a pH of 11 was obtained. Immediately thereafter hydrochloric acid was added until the pH was reduced to approximately 7 after which the mixture was frozen and lyophilized. A yield of 20 g of light yellow colored fluffy product which was soluble in water or buffer solution of pH 7.0 to the extent of 10% was obtained. Several other preparations were made in which the ratio of gossypol to peanut protein was varied. When large quantities of this preparation are required it is of advantage to precipitate the gossypol-protein product with hydrochloric acid by lowering the pH to 4.5. This product is removed by centrifugation and can then be transferred with a small amount of water to the lyophilizing apparatus and dried. In order however to effect solution of the dry product in water it is necessary to adjust the pH to 7 with alkali. Water-soluble gossypol-glycine and gossypol-lysine products were prepared by combining equal weights of gossypol and the amino acids according to the above mentioned procedure. Similar procedures were used in preparing combination products with dextrose and starch.

*Physiological experiments.* Two to 8 goldfish were placed in the test and control solutions contained in wide-mouthed glass jars 6½" high and 6" in diameter. Each 2 fish were in approximately one liter of solution. Air was bubbled through each solution at the same rate, the pH was adjusted to 7.0 in each case, and the temperature was maintained at 75° F. The time of exposure of the fish to the test solutions until death ensued has been recorded as the average value of the time taken for the death of all experimental animals in a test solution. The range of variation of death time in any given solution did not exceed 20%.

*Testing of crystalline gossypol.* A mixture of gossypol in water at a concentration of 0.04% gossypol and adjusted to pH of 7.0 was violently agitated. Nevertheless the gossypol remained upon the surface of the water after agitation had ceased. Goldfish placed in this test solution lived for seven days and upon transfer to fresh water continued to be healthy. Combination-product formation was suggested by the following experiment. Gossypol was added to a .005% solution of peanut protein and the mixture was stirred. The pH of the solution was increased to 10.3 by use of dilute sodium hydroxide solution and then reduced to neutrality by use of hydrochloric acid. Under such conditions of preparation, the gossypol remained in solution throughout the test and in a concentration range of 1 to 5 parts per 100,000 parts of 0.005% protein solution was quite lethal to fish within 24 hours. Control solutions containing an equivalent amount of protein and sodium chloride were not harmful to the test animals. Physical suspension of the gossypol does not put it in a condition wherein it is toxic to fish. It is necessary to completely solubilize the gossypol and maintain it in solution with protein in order to obtain the toxic effect.

*Water-soluble combination products of gossypol.* In Table I are shown the extractable gossypol contents of each of the freshly prepared samples of combination products and their toxicities to aquarium fish. An aqueous solution of gossypol-peanut protein that produced death of fish within 1 hour at a concentration of 1 part of the material per 20,000 parts of water was stored for 48 hours at 38° F. After this period the solution still had its original toxicity and gossypol content. Control solutions containing an equivalent amount of protein, amino acid, starch, dextrose, and sodium chloride were not harmful to the test animals.

At a concentration of one part of pigment glands to 2000 parts of water, the glands were lethal to the fish. However, such solutions had only about one-half of the toxicity of solutions of combination product of gossypol which were of the concentration of one part of product to 20,000 parts of water. Results of these investigations are shown in Table II.

It is quite clear from a comparison of the results in Tables I and II that the combina-

TABLE I.
Toxicities to Goldfish of Water-soluble Combination Products of Gossypol with Materials of Natural Origin.

| Grams of combination product per ml aquarium bath | Combination product: Gossypol-peanut protein A | Gossypol-peanut protein B† | Gossypol-peanut protein C‡ | Gossypol-casein | Gossypol-starch | Gossypol-dextrose | Gossypol-glycine | Gossypol-lysine |
|---|---|---|---|---|---|---|---|---|
| | Extractable gossypol content of dry product,* % | | | | | | | |
| | 46.1 | 40.0 | 52.2 | 41.0 | 36.9 | 39.2 | 28.4 | 44.8 |
| | Exposure of fish to test sol. before death —(hr) | | | | | | | |
| $5 \times 10^{-5}$ | 1. | 1.7 | 1.3 | 2. | 1.4 | 1.7 | 1.7 | 2. |
| $2.5 \times 10^{-5}$ | 1.7 | 4.4 | 1.6 | <24. | 2.3 | 2.4 | 3.4 | <24. |
| $1.6 \times 10^{-5}$ | 3.6 | 6.1 | 3.1 | <24. | — | 2.9 | 2.7 | <24. |
| $1.25 \times 10^{-5}$ | 4.0 | <24. | <24. | <24. | — | — | <24. | <24. |
| $1 \times 10^{-5}$ | <24. | <24. | <24. | <24. | — | — | <24. | >24. |

* Calculated on dry-weight basis. Percent extractable gossypol determined by application of the antimony trichloride spectrophotometric method applied to chloroform solutions prepared from aqueous ethanol extracts (8).

† This sample was lyophilized at pH 4.5; all other samples in Table I were lyophilized in aqueous solution of pH 7.0.

‡ Two parts of gossypol were combined with one part of protein; in all other samples in Table I, gossypol was combined with an equivalent weight of material of natural origin.

TABLE II.
Toxicities of Separated Cottonseed Pigment Glands to Goldfish.

| Lot of pigment glands | Extractable pigment content of glands*: Gossypol,† % | Gossypurpurin,‡ % | G of pigment glands per ml of aquarium bath sol. | Exposure of fish to test sol. before death (hr) |
|---|---|---|---|---|
| A | 39.6 | 0.83 | $5 \times 10^{-4}$ | 3.9 |
| | | | $2.5 \times 10^{-4}$ | 5.3 |
| | | | $1.7 \times 10^{-4}$ | <24.0 |
| B | 33.8 | 1.74 | $5 \times 10^{-4}$ | 6.3 |
| | | | $2.5 \times 10^{-4}$ | <24.0 |
| C | 29.9 | 1.16 | $5 \times 10^{-4}$ | 5.7 |
| | | | $2.5 \times 10^{-4}$ | <24.0 |

* Calculated on basis of weight of pigment glands.

† Determined by application of the antimony trichloride spectrophotometric method to chloroform solutions prepared from aqueous ethanol extracts of the glands (8).

‡ Calculated on basis of $E^{1\%}_{1\ cm}$ at 568 $m_\mu$ of chloroform extracts of glands (3).

tion products of gossypol are much more toxic to goldfish than the pigment glands. Although the gossypol in pigment glands is not as active toward goldfish as that in the combination products it is much more active than pure gossypol itself. This observation would indicate that gossypol, as it exists in the pigment glands, is in combination with some of the other material in the glands rather than completely in the free state. Such combination is of a nature that renders the gossypol either completely soluble or at least dispersible in a very finely divided state in aqueous medium.

*Discussion.* The exact nature of the reaction of gossypol with the materials used in making the water-soluble products is not yet known. The fact that gossypol contains hydroxy groups and carbonyl groups would indicate that this material is capable of combining with the amino groups of proteins and amino acids, and the hydroxy groups of carbohydrates. It is known that when cottonseed meal is cooked and the gossypol re-

8. Hall, C. M., Castillon, L. E., Guice, W. A., and Boatner, C. H., *J. Am. Oil Chem. Soc.*, 1948, v25, 457.

leased from the pigment glands, some of it reacts with the reactive groups in the meal and is "bound." Under such circumstances the gossypol is detoxified. For the combination products described in this publication the gossypol is rendered more toxic by a process which maintains it in solution. A more thorough study of the reactions of gossypol with amino acids, proteins, and carbohydrates, and of the effect of storage and heat on such products would undoubtedly clarify the nature of this combination product and probably throw light on the reaction that takes place when cottonseed or cottonseed meats are cooked in order to detoxify the gossypol.

*Summary.* (1) Gossypol was combined with proteins, amino acids, and carbohydrate materials by a procedure involving mixing of the gossypol and other combining substances in alkaline solutions and subsequent lyophilization of the neutralized frozen solutions. (2) Crystalline gossypol when added to the aquarium bath had no visible effect upon goldfish. The water-soluble combination products of gossypol were toxic to fish in concentrations as small as one part of gossypol product to a hundred thousand parts of water. (3) Separated pigment glands were toxic to fish but were significantly less toxic than the water-soluble combination products of gossypol.

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## Lipotropic Effects of Liver Extract, Vitamin $B_{12}$ and Choline.* (17997)

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It has been reported that crude liver extract exerts a lipotropic effect and will prevent fibrosis of the liver when administered to rats receiving a high fat diet(1). The lipotropic effect of the liver extract did not seem to be due to choline content. In fact, other supplements which did not give protection against dietary induced liver injury had a higher content of choline than was present in liver extract(2). In further investigations a vitamin $B_{12}$ concentrate, prepared from liver, also exerted a definite effect in preventing accumulation of fat in the liver of rats(3). In the present study the relationship between dosage of liver extract and lipotropic effect was studied. Observations were also made on the possible lipotropic effect of combinations of choline, inositol and folic acid, which are present in small amounts in liver extract and vitamin $B_{12}$ concentrate. Crystalline vitamin $B_{12}$ alone and in combination with small amounts of choline was also observed for lipotropic activity.

*Methods.* Male Sprague-Dawley rats weighing between 120 and 150 g were used. They were fed the high-fat diet (51% lard) and the control diet (6% lard) recently described(1). These diets were fed for the periods listed in the tables. When the animals were placed on the synthetic diet, injections of the supplements were begun. All supplements were administered subcutaneously except in one experiment with liver extract as noted in Table I. At the end of the study sections were taken from the left lobe of the liver and stained with hematoxylin and eosin or with Sudan III for fat. The remainder of the liver was analyzed for total

* The authors wish to thank Eli Lilly and Co. for the liver extract, Merck and Co. for the crystalline vit. $B_{12}$ and Mead Johnson and Co. for the Oleum Percomorphum.

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